Risk factors and biomarkers for malignant mesothelioma

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DOI (link to publication from Publisher):
10.54337/aau307981410

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

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DISSERTATION SUBMITTED 2019

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted in 2019

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Dissertation submitted: 08.03.2019

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ISSN (online): 2246-1302
ISBN (online): 978-87-7210-406-5

Published by:
Aalborg University Press
Langagervej 2
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2019

This thesis is based on the following manuscripts:

1. Panou V, Vyberg M, Weinreich UM, Falkmer U, Røe OD. **The established and future biomarkers of malignant pleural mesothelioma.** *Cancer Treat Rev. 2015;41(6):486-495*.

2. Panou V, Gadiraju M, Wolin A, Weipert CM, Skarda E, Husain AN, Patel JD, Rose B, Zhang SR, Weatherly M, Nelakuditi V, Johnson AK, Helgeson M, Fischer D, Desai A, Sulai N, Ritterhouse L, Røe OD, Turaga KK, Huo D, Segal J, Kadri S, Li Z, Kindler HL, and Churpek JE. **Frequency of Germline Mutations in Cancer Susceptibility Genes in Malignant Mesothelioma.** *J Clin Oncol. 2018;36(28):2863-2871*.

3. Panou V, Vyberg M, Meristoudis C, Hansen J, Bøgsted M, Omland Ø, Weinreich UM, Røe OD. **Non-occupational exposure to asbestos is the main cause of malignant mesothelioma in women in North Jutland, Denmark.** *Scand J Work Environ Health* 2019;45(1):82-89.

4. Panou V, Petersen T, Weinreich UM, Vyberg M, Meristoudis C, Hansen J, Bøgsted M, Omland Ø, Røe OD. **The major role of non-occupational asbestos exposure in inducing malignant mesothelioma in Aalborg, Denmark**. *Occupational & Environmental Medicine* (submitted).

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2. Hassan R, Morrow B, Thomas A, Walsh T, Lee MK, Gulsuner S, Gadiraju M, Panou V, Gao S, Mian I, Khan J, Raffeld M, Patel S, Xi L, Wei JS, Hesdorffer M, Zhang J, Calzone K, Desai A, Padiernos E, Alewine C, Schrump DS, Steinberg SM, Kindler HL, King MC, Churpek JE. Inherited Predisposition to Malignant

Mesothelioma and Survival Following Platinum Chemotherapy. *Proceedings of the National Academy of Sciences* (in press).

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2. Panou V, Gadiraju M, Wolin A, Weipert CM, Skarda E, Husain AN, Patel JD, Rose B, Nelakuditi V, Johnson AK, Helgeson M, Fischer D, Sulai N, Turaga K, Huo D, Segal J, Kadri S, Li Z, Kindler HL, Churpek JE. Frequency of germline mutations in cancer susceptibility genes in malignant mesothelioma. Conference abstract presented at the American Society of Clinical Oncology (ASCO) Annual Meeting 2018 (*Journal of Clinical Oncology* 36, 2018, suppl; abstr 8564).

3. Panou V, Meristoudis C, Weinreich UM, Røe OD, Vyberg M. Impact of Calretinin immunohistochemistry and the iMig guidelines in the malignant mesothelioma diagnosis. Conference abstract presented at the 14th International Mesothelioma Interest Group Conference 2018.

4. Røe OD, Omland Ø, Panou V, Wannag A. Asbestos Consumption, Eternit and Pleural Mesothelioma in Norway and Denmark: Similar Populations, Different Stories. Conference abstract presented at the 14th International Mesothelioma Interest Group Conference 2018 (Best Poster Award).

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7. Bak J, Panou V, Weinreich UM, Røe OD. Malignant mesothelioma in males in Northern Jutland, Denmark- incidence, diagnosis and survival. Conference abstract presented at the 48th Nordic Lung Congress 2017.

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9. Panou V, Vyberg M, Meristoudis C, Røe OD. No BAP-1 cancer syndrome associated malignancies detected among 558 Danish patients with malignant mesothelioma. Conference abstract presented at the 13th International Mesothelioma Interest Group Conference 2016.

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ENGLISH SUMMARY

Malignant Mesothelioma (MM) is an aggressive malignancy with limited therapeutic options, firmly associated with asbestos inhalation. It presents typically in the pleura (MPM), infrequently in the peritoneum (MPeM), and rarely in pericardium and tunica vaginalis. Three major histopathological subtypes are described, epithelioid, sarcomatoid and biphasic. Both occupational and non-occupational exposure to asbestos can cause MM. However, the latter is under-reported and under-investigated, mostly due to the rarity of the disease. It is suspected that cancer susceptibility genes are also involved in the MM genesis but their spectrum and prevalence are unknown, with the sole exception of the well-described *BRCA1–associated protein 1* (*BAP1)* gene. The North Denmark Region in Denmark has an extraordinary high MM incidence as a result of two large asbestos emitting industries that operated in its capital in densely populated areas and employed more than 25,000 workers for more than six decades. This thesis is based on four studies that overall aim to investigate risk factors for MM, specifically non-occupational asbestos exposure and genetic susceptibility, and to outline the most important current and future MM biomarkers.

The first study summarizes the established and most promising future biomarkers in the diagnosis, prognosis and prediction of MPM, emphasizing the need of improved biomarkers that can be more helpful in all clinical contexts. The second study demonstrates that non-occupational asbestos exposure has had a major impact on the men and women of the North Denmark Region, as it is the main cause of MM for women and it is implicated in the MM pathogenesis for the majority of the men. The study also identifies a ´´hotspot´´, where the population is at higher risk of MM. Furthermore, it shows that male and female MM patients have different asbestos exposure profiles, with the most common exposure types being domestic and/or environmental for the women and occupational and/or environmental for the men. The study also indicates that the epithelioid subtype is associated with non-occupational and the non-epithelioid with occupational asbestos exposure. Furthermore, MPeM is overrepresented among women compared to men and it is more frequent among women with occupational versus non-occupational asbestos exposure. The third study describes 13 genes with germline mutations in 23/198 MM patients, *BAP1*, *BRCA2*, *CDKN2A, ATM, BRCA1, TP53, MSH6, TMEM127, CHEK2*, *MRE11A, VHL, WT1*, and *SDHA*. Six of these genes are overrepresented in an MM versus a non-cancer population (*BAP1, BRCA2, CDKN2A, TMEM127, VHL* and *WT1*). Clinical predictors of inherited mutation include peritoneal disease, limited asbestos exposure, second cancer diagnosis and younger age. Finally, the study unmasked that most of the mutated genes are involved in the homologous recombination DNA repair pathway.

In conclusion, this thesis assesses two under-investigated risk factors of MM, genetic susceptibility and non-occupational asbestos exposure, and outlines the most important MM biomarkers. The thesis provides a framework for future studies.

DANSK RESUME

Malignt mesotheliom (MM) er en aggressiv malignitet, der er stærkt forbundet med asbest eksponering. Det forekommer typisk i pleura (MPM) og sjældent i peritoneum (MPeM), perikardium og tunica vaginalis. Tre histopatologiske subtyper er beskrevet, epithelioid, sarcomatoid og bifasisk. Både erhvervsmæssig og ikke-erhvervsmæssig eksponering for asbest kan forårsage MM. Imidlertid er MM som følge af ikke-erhvervsmæssig asbesteksponering underrapporteret og der er få studier omkring det, hovedsagelig på grund af sygdommens sjældenhed. Der mistankes også, at germline mutationer er involveret i MM genesen, men deres spektrum og forekomst er ukendte, undtaget det velbeskrevne *BRCA1–associated protein 1 (BAP1)* gen. Region Nordjylland i Danmark har en ekstraordinær høj MM-forekomst som følge af to store asbestindustrier, der lå centralt i Aalborg og beskæftigede flere end 25.000 arbejdere i mere end seks årtier. Denne afhandling er baseret på fire studier, som undersøger risikofaktorer for MM, især ikke-erhvervsmæssig asbesteksponering og genetisk disposition, og skitserer de vigtigste nuværende og fremtidige MM biomarkører.

Det første studie opsummerer de etablerede og mest lovende fremtidige biomarkører i diagnosen og prognosen samt prædiktion af MPM behandlingseffekt. Det andet studie viser, at ikke-erhvervsmæssig asbest eksponering har haft stor indflydelse på udvikling af MM blandt nordjyske mænd og kvinder, da det er hovedårsagen til MM for kvinder, og det er impliceret i MM patogenesen for flertallet af mænd. Studiet identificerer også et "hotspot ", hvor befolkningen har større risiko for MM. Desuden viser studiet, at mandlige og kvindelige MM patienter har forskellige eksponeringsprofiler for asbest; de fleste kvinder har været husstands- og/eller miljø udsatte for asbest, mens mænd hovedsageligt har haft erhvervs- og/eller miljømæssig asbest eksponering. Endelig indikerer studiet, at epithelioid MM er forbundet med ikke-erhvervsmæssig og ikke-epithelioid MM med erhvervsmæssig asbesteksponering. Endvidere er MPeM hyppigere forekommende blandt kvinder sammenlignet med mænd, og blandt kvinder med erhvervsmæssig versus ikke-erhvervsmæssig asbesteksponering. Det tredje studie beskriver 13 gener med arvelige mutationer hos 23/198 MM patienter (*BAP1, BRCA2, CDKN2A, ATM, BRCA1, TP53, MSH6, TMEM127, CHEK2, MRE11A, VHL, WT1* og *SDHA*). Seks af disse gener er overrepræsenteret i en MM versus en ikke-kræftpopulation (*BAP1, BRCA2, CDKN2A, TMEM127, VHL* og *WT1*). Kliniske markører for en arvelig mutation indbefatter MPeM, ingen asbesteksponering, anden kræftdiagnose og yngre alder. Endelig afslører studiet, at de fleste muterede gener er involveret i DNA reparation, den homologe rekombinations-signalvej.

Afslutningsvis omhandler denne afhandling to risikofaktorer for MM, genetisk modtagelighed og ikke-erhvervsmæssig asbesteksponering, og beskriver de vigtigste MM biomarkører. Endelig sætter resultaterne i denne afhandling ramme for fremtidige studier.

ACKNOWLEDGEMENTS

A lot of people have contributed to this thesis, both colleagues, collaborators, friends and family. First and foremost, my main supervisor, **Ass. Prof. Oluf Dimitri Røe**; his expertise and devotion in research in malignant mesothelioma generated the whole project in the first place. You have been not only a mentor to me but also a dear friend, whose profound knowledge, guidance and creativity have been essential to this PhD. I feel very fortunate that I have met you, worked with you and I look forward to many years of future collaborations. Similarly, I want to thank my co-supervisor, **Ass. Prof. Ulla Møller Weinreich**, who has been a tremendous help for me in every step of this process and has greatly contributed to this thesis and to my training as a researcher; you are and have always been an inspiration to me. Heartfelt thanks must also be given to the other two co-supervisors, **Prof. Ursula Falkmer** and **Prof. Martin Bøgsted**, for their significant part in this work, their valuable comments and the constructive criticism.

I would like to express my gratitude to **Lene Birket-Smith**, **Carl Nielsen** and **Carl-Otto Gøtzsche** for believing in me and for financing my research; I would not be able to do any of this work if you hadn´t granted me this possibility. My co-authors deserve a big thank for their significant contribution to the four papers that compose this thesis. **Prof. Mogens Vyberg** for his inestimable expertise and his priceless input on every aspect of this thesis, **Christos Meristoudis** for his extraordinary work with the tumor samples, **Johnni Hansen**, the expert of the Danish Registries, **Prof. Øyvind Omland** for his valuable insight into epidemiological and asbestos exposure matters and the very talented young researcher, **Thomas Ringgaard Petersen**. Furthermore, I am extremely grateful to **Prof. Hedy Kindler**, **Ass. Prof. Jane Churpek** and **Dr. Kiran Turaga** for giving me the opportunity for a very fruitful and inspiring research stay at The University of Chicago, for involving me in a truly interesting project and for their invaluable supervision; my research stay with you has had a big impact on my researcher education. Lastly, I would like to acknowledge all the collaborators from The University of Chicago, whose hard work resulted in an important scientific publication.

I am also indebted to my collaborators from different departments and institutions. Firstly, a special thank goes to the most competent research nurses, **Mie Ravn**, **Hanne Bormann Larsen**, **Lillian Skov Søndergaard Lundberg**, **Rikke Bækkely Sass Mathiesen** and **Dorthe Brønnum**; secondly, to the most amazing secretaries I know, **Lisbeth Gadegaard, Marianne Ferch Helledie, Lise Larsen, Maria Lund Nielsen, Sanne Andersen, Karina Colstrup Ibsen**; and to all my wonderful colleagues at the Department of Respiratory Diseases, Aalborg University Hospital. I would also like to thank the Meso-HUNT group for fantastic teamwork, and especially **Rana Bibi, Vitas Nekrasas**, **Annette Pedersen**, **Jette Simoni**, **Louise**

Serup Christoffersen, Malene Saxberg, Anne Bentzen-Petersen, Peter Bach, Søren Thomsen and **Benedict Kjærgaard**. In addition, I want to acknowledge the doctors, nurses, secretaries and laboratory staff from the Institute of Pathology, the Department of Thoracic Surgery, the Department of Oncology and the Occupational Clinic at Aalborg University Hospital for their assistance with the studies. Lastly, I owe gratitude to all the participating patients that despite their diagnosis with a fatal malignancy, they selflessly decided to be a part of a research project in order to help the future patients.

I am deeply thankful to all my friends in Greece, Denmark and abroad; you tolerate me and you are always there when I need a good laugh or a shoulder to cry on. I want to wholeheartedly thank my family and particularly my sister and best friend in the whole world, **Angeliki,** my forever little brother, **Kostis,** and my cousin/extra sister, **Konstantina**, for their love and for, come what may, undoubtedly being there for me; I am privileged to have you in my life! From the bottom of my heart, I thank my life companion, **Evgenios**, for his love, patience, understanding and encouragement; even in the darkest moments you can make me smile and you never stop believing in me.

I dedicate this work to my biggest fans, my parents, **Andreas** and **Persefoni**, for their unconditional love and endless support. There are not enough words in this world to express how thankful I am for all that you have offered me- Thank you!

Vasiliki Panou, Aalborg 2019

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ABBREVIATIONS

MM:	Malignant Mesothelioma
MPM:	Malignant Pleural Mesothelioma
MPeM:	Malignant Peritoneal Mesothelioma
km:	kilometers
SV40:	Simian Virus 40
NF- κB:	Nuclear Factor κ-light chain enhancer of activated B-cells
BAP1:	BRCA1–associated protein 1
CT:	Computed Tomography
PET/CT:	Positron Emission Tomography
FDG:	2-[fluorine 18]Fluoro-2-deoxy-D-Glucose
WT1:	Wilms tumor protein-1
WT:	Wilms tumor
EpCAM:	Epithelial Cell Adhesion Molecule
OS:	Overall Survival
EPP:	Extra-Pleural Pneumonectomy
P/D:	Pleurectomy/Decortication
HIPEC:	Hyperthermic Intraperitoneal Chemotherapy
EGFR:	Epidermal Growth Factor Receptor
PD-L1:	Programmed Death Ligand 1
HMGB1:	High-Mobility Group Box 1
SOMAmers:	Slow Off-Rate Modified Aptamers
DAF:	Danish Asbestos Cement Factory
kg:	kilograms
ADCA:	Adenocarcinoma
CEA:	Carcinoembryonic Antigen
CK5:	Cytokeratin 5
CL4:	Claudin-4
CR:	Calretinin
ER:	Estrogen Receptor alpha
MG:	Mammaglobin
PDP:	Podoplanin
TTF1:	Thyroid Transcription Factor-1,
lncRNA:	long non-coding RNA
SMRP	Serum Mesothelin Related Protein
SD:	Standard Deviation

IQR:	Interquartile Range
RR:	Relative Risk
CI:	Confidence Interval
OR:	Odds Ratio
ExAC:	Exome Aggregation Consortium
FDR:	First Degree Relatives
SDR:	Second Degree Relatives
HR:	Homologous Recombination
VUS:	Variant of Uncertain Significance
PARPi:	Poly(ADP-ribose) polymerase inhibitors

CHAPTER 1. BACKGROUND

Diffuse Malignant Mesothelioma (MM) is a rare and aggressive malignant neoplasm caused mainly by asbestos inhalation (1). This neoplasm derives from the mesothelial and submesothelial cells of serosal surfaces and presents in the pleura (MPM) in 80-90% of the cases, in the peritoneum (MPeM) in 10-15% and rarely (less than 1%) in the pericardium and tunica vaginalis testis (1–3). There are three histopathological MM subtypes, the epithelioid, which is the most common and has the most favorable prognosis, the more aggressive sarcomatoid and the biphasic subtype, that consists of both sarcomatoid and epithelioid components (3,4). Two subtypes of borderline malignant potential have also been identified, the well-differentiated papillary mesothelioma and the benign multicystic mesothelioma, which are sporadic and mostly develop in the peritoneum (3). The parameters that influence the development of the MM subtypes (epitheliod, sarcomatoid or biphasic) and MM location (pleura, peritoneum, pericardium or tunica vaginalis testis) are unknown.

1.1. EXPOSURE TO ASBESTOS AND ERIONITE

Asbestos is a set of six minerals classified in two principal groups, the amphiboles, consisting of crocidolite, amosite, tremolite, actinolite and anthophyllite and the serpentines that has one compound, chrysotile (5). The amphiboles are characterized by straight, longer fibers, while chrysotile is more flexible and has curly, short fibers (5) (Figure 1-1.1) (1). The word asbestos comes from the Greeks and it means ´´inextinguishable, unquenchable´´, characterizing the material's fire resistance, durability and flexibility (1). Asbestos was used by various cultures since prehistoric times; asbestos fibers have been found in debris from settlements from the Stone Age, in lamps and candles as early as 4000 B.C., and in Finnish pottery 4,500 years ago (1,6,7). Modern asbestos history can be traced around 1850, starting from Canada and South Africa (6). The following decades, asbestos mining and manufacturing exploded, as manufacturers became fully aware of its desirable physical properties and marketed asbestos as the ´´magic mineral´´ (1,6,7). It has since then been used for insulation of pipes, buildings, shipbuilding, car brakes, adhesives, and even toys, jewelry, textiles, and cigarette filters (1).

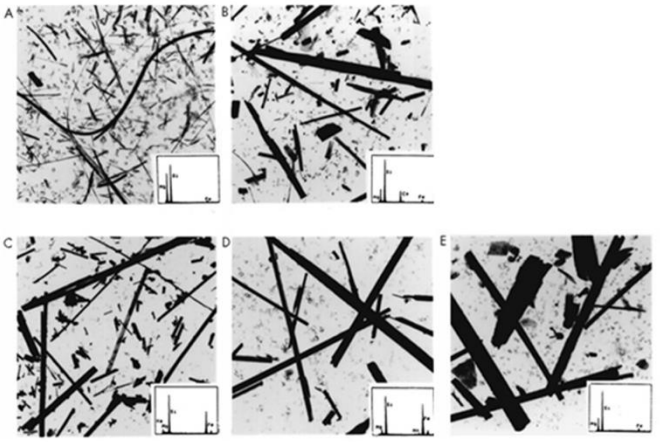


Figure 1-1.1 Types of asbestos illustrated on election microscopy with energy dispersive X-ray spectra (on a 5µm scale). A: Chrysotile, B: Tremolite, C: Crocidolite, D: Amosite, E: Anthophylite. Reproduced with permission from Encyclopedia of Occupational Health and Safety, Jeanne Mager Stellman, Editor-in-Chief. International Labor Organization, Geneva. © 2011, ´´Asbestos-Related Diseases´´ by Margaret Becklake.

The direct causal relation between asbestos and MM was first documented in 1960 by the South African pathologist John Christopher Wagner, who observed a high incidence of this rare malignancy among the workers at the mines of the North-Western Cape province (8). By the end of the 1960s, there were published more than 200 reports from several parts of the world, such as the crocidolite mines of Perth and the asbestos industries in the United Kingdom and the United States of America (USA), identifying asbestos as a carcinogen (6,7). For several years, chrysotile was considered to be less carcinogenic than the amphiboles by some scientists, while other researchers claimed that chrysotile can only lead to MM when contaminated with amphiboles (9,10). Such research has enabled the asbestos industry to keep using chrysotile for several decades after it was first linked to MM and other malignancies (1). Nonetheless, several animal models and epidemiological studies have concluded that chrysotile is an important risk factor for MM and all types of asbestos are declared as carcinogens by the World Health Organization and the International Agency for Research on Cancer (1). Asbestos is also an important risk factor for lung cancer and the World Health Organization estimates that for every new case of MM, there are six asbestos related cases of lung cancer (11,12). Gastric, larynx, colorectal and ovary cancer are associated with asbestos inhalation, as well (11).

Asbestos was banned in most Western countries in the span from 1970-2005, with the exception of the USA and Canada, as asbestos is only partly banned in the USA, and it was only banned in Canada in 2018 (13,14). In Denmark, asbestos was partly

banned in 1980, further strict restrictions were adopted in 1986 and asbestos was completely banned in 1988 (15). However, asbestos use and mining is still to be prohibited in developing countries, and worldwide asbestos production is approximately 2.2 million metric tons per year, with heavily populated countries leading the world mine production (Figure 1-1.2) (16).

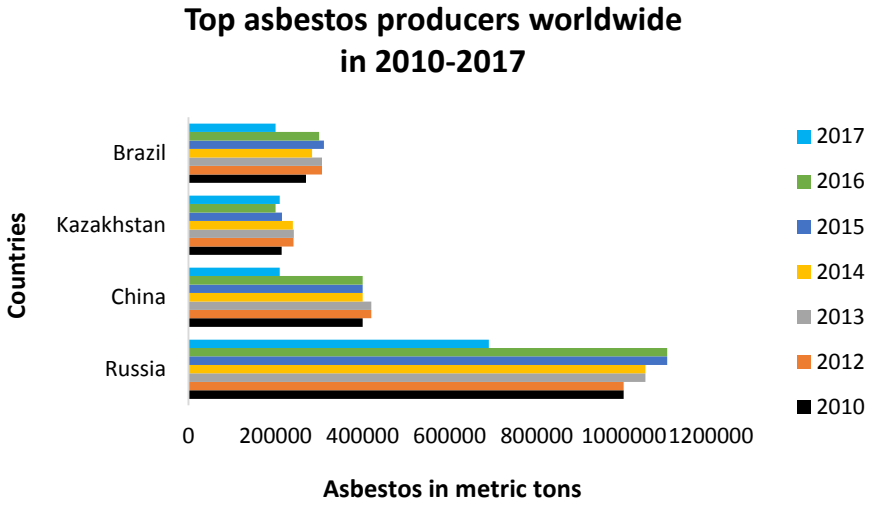


Figure 1.1-2. The biggest asbestos producers worldwide and their asbestos production during 2010-2017 based on the existing literature (5,6,17–19)

Not only occupational, but also non-occupational exposure to asbestos causes MM. Non-occupational exposure includes domestic and environmental exposure, as well as exposure to natural occurring asbestos or to commercial asbestos-containing materials (1,20–22). Family members that live under the same roof with asbestos workers can be exposed to asbestos domestically through the fibers that are transported by the laborers on their clothes (23). The extent of the contamination of the worker's clothing is highly dependent on his/hers tasks and the precaution measures that are followed, but even low-scale exposure to asbestos can cause MM (24). In fact, there is no threshold under which asbestos use is safe, and no linear dose–response relationship between asbestos exposure and MM (14). Some individuals are, though, more susceptible to MM after exposure to asbestos than others, probably depending on genetics, the duration of the exposure and the age at first exposure (14).

Environmental exposure to asbestos is possible via airborne contamination of residential areas due to the distribution of asbestos-laden materials and waste products from local asbestos plants (25,26). Studies have shown increasing risk of MM with decreasing distance from an asbestos plant, with the risk being higher in a 10km radius from asbestos industry (20). However, the quantification of the MM risk attributable

to non-occupational exposure is challenging and there is limited research on this matter (27).

Despite the ban of asbestos in the Western countries, individuals may still be exposed to it due to legacy of past use. Asbestos has been a popular material and as a result more than 3,000 products were registered to contain asbestos in the 1970´s (1). The linkage between exposure to these profoundly different products and risk of MM is difficult to assess. Furthermore, asbestos products are still in place in a large part of public and private buildings constructed after the second world war and up to the 90´s (5,28,29). The asbestos dust from manipulation of asbestos-containing building materials is potentially compromising to the individual's health, but the personal risk for the malignancy is difficult to determine (30,31).

Naturally occurring asbestos and asbestos-like fibrous minerals can also be carcinogenic when found in residential areas and aerosolized due to natural dust emissions or anthropogenic activity (22,32). Natural occurring tremolite and chrysotile has been identified in villages in Turkey, Greece, Corsica, Cyprus, and New Caledonia, fluoroedenite in Sicily, crocidolite in southwestern China and erionite in Cappadocia and in the USA (33–38). Erionite is shown to be more potent than asbestos in causing MM (39,40). In these areas, the MM incidence rates were found to be 100 to 800 times higher than global background rates, the male:female ratio was close to one, and age at onset was younger than observed in occupationally exposed populations.

1.2. RARE CAUSES OF MALIGNANT MESOTHELIOMA

Patients that have been exposed to Thorotrast, a radiographic contrast material that was used in the 1950´s and atomic energy workers chronically exposed to lower levels of radiation are also in high risk for developing MM (41). There are also studies describing an association between MM and radiation treatment for Non-Hodgkin lymphoma and testicular cancer, as a statistically significant excess of MM was identified for these patients (42,43). Simian Virus 40 (SV40) is a DNA tumor virus that contaminated some stocks of polio vaccines between 1954-1961 (2). SV40 has also been shown to induce MM in animals and it has been implicated in human MM tumorigenesis (44). Carbon nanotubes is a family of nanoconstructed materials that are being used in electronics, heating elements, batteries and energy storage, fibers and fabrics, catalyst supports, air and water purification, dental implants, targeted drug delivery, and other medical applications (1). It has been suggested that carbon nanotubes can behave like asbestos fibers and induce carcinogenicity (45–47). Future research is needed in order to understand their potential role in causing MM and to achieve risk control.

1.3. EPIDEMIOLOGY

The incidence of MM varies considerably internationally, and it seems to be dependent on the asbestos exposure burden of every country. The incidence is increasing and is expected to continue to rise by minimum 5-10% per year until 2025 in the western countries (40,48). In the USA the incidence of MM is estimated to be between 1–2/million in states with minimal and 10–15/million in states with large exposure to asbestos (40,49). The age-adjusted MM incidence in Europe during 1994-2008 has been reported to be 10-30/million, with Italy, United Kingdom and West Germany being three of the biggest centers for industrial use of asbestos in the 20th century (49,50). There are, though, vast differences in MM incidence, not only among the European countries but also within each country´s borders (50). Australia has one of the highest global age-standardized incidence rates for MM of 30/million due to heavy asbestos industry and asbestos mines that operated for several decades (49,51). There are unfortunately few reliable registry data about asbestos exposure and MM incidence and mortality from the developing countries.

MM has a long latency from first exposure to MM diagnosis varying between 20-70 years (49). Pleural disease has been reported to have longer latency than peritoneal (52). MPeM has also been linked to heavier asbestos exposure, both in terms of type of asbestos fibers, chrysotile or amphiboles, and type of exposure, occupational and non-occupational (53). Researchers have claimed that the attributable risk of MPeM is higher as a result of amphiboles compared to chrysotile and occupational versus non-occupational asbestos exposure (53). It has also been suggested that the risk of MPeM increases remarkably for asbestos workers with high cumulative exposure in comparison to MPM (40,53). However, there are several cases of MPeM attributed to chrysotile asbestos and non-occupational exposure in the literature, as well as studies that demonstrate that MPeM is more common among individuals with no asbestos exposure (40). The above divergent conclusions are a result of the limited research of MPeM due to the low incidence rates of the disease worldwide.

MM is more prevalent in men than women with a 2-5:1 ratio, which has been ascribed to their higher degree of occupational exposure to asbestos (40,54). There have been studies presenting asbestos exposed female cohorts, e.g. in connection with whitewashing of houses in Metsovo, Greece, and in these cases it was the women that primarily developed the malignancy (55). Several studies describe a much lower percentage of female MM patients with known asbestos exposure in comparison to their male counterparts (40). However, it is not clear if non-occupational asbestos exposure was taken into consideration in these studies and in which extent. It has also been suggested that men and women could develop different phenotypes of MM, as female MM patients have a favorable survival compared to men (56). The lack of large studies comprising of women with MM challenges the further investigation of these matters. Furthermore, MPeM has a weaker causal link to asbestos for women

than men, but the mechanisms involved in the MPeM tumorigenesis are not fully known (40,53).

1.4. PATHOGENESIS AND MOLECULAR PROFILE

The most common location for MM development is the pleura. The pleura is a membrane that covers the inner surfaces of the chest cavity and consists of a layer of mesothelial cells supported by a network of connective and fibroelastic tissue (1). The visceral pleura lines the lung, whereas the parietal pleura lines the costae, the diaphragm, and the mediastinal structures (Figure 1.4-1) (1). The mesothelial cells provide a non-adhesive and protective surface, and they are primarily involved in cell and fluid transport across the serosal cavities, but they have also a plethora of functions, among others inflammatory responses and phagocytosis of fibers (1). Asbestos fibers get transported to the pleura through inhalation, and their shape, especially their length/width ratio is important as to the depth of the lung penetration (2). There are different pathways, in which asbestos can induce MM. Firstly, asbestos fibers cause irritation in the pleura and disrupt the mitotic process, which can lead to aneuploidy and chromosome damage typical for MM (2). Asbestos-exposed mesothelial cells and macrophages release a variety of cytokines and growth factors, which induce inflammation and tumor promotion, including tumor necrosis factor-α, interleukin-1β, transforming growth factor-β and platelet-derived growth factor (57). Furthermore, asbestos triggers the generation of iron-related reactive oxygen species that cause DNA damage and stand breaks (2). Moreover, asbestos induces phosphorylation of the mitogen-activated protein kinases and of extracellular signal–regulated kinases 1 and 2, which increases the expression of early-response proto-oncogenes in mesothelial cells (2).

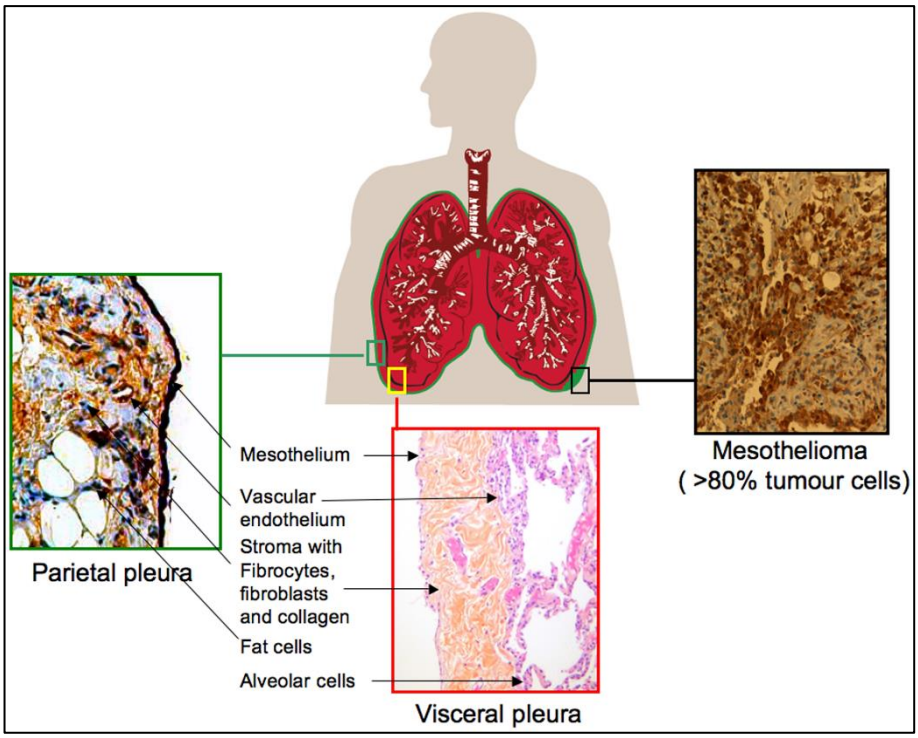


Figure 1.4-1. Representation of the normal parietal pleura, the visceral pleura and pleural mesothelioma with the most abundant cell types. Reproduced from (1) with permission from the publisher.

As a consequence of the above-mentioned mechanisms, cytotoxicity, DNA damage, frustrated phagocytosis and chronic inflammation are caused in the pleura and result in functional abnormalities conveyed by gene, microRNA and protein expressions. Numerous studies have demonstrated that MM has a highly complex and variable molecular profile among patients (1,58). Loss-of-heterozygosity investigations have demonstrated repeated deletions of distinct sites within chromosome arms 1p, 3p, 6q, 9p, 13q, 15q and 22q, with the most commonly transformed locations being the tumor suppressors *CDKN2A*–ARF at 9p21, and *NF2* at 22q12 (1). Nuclear factor κ-light chain enhancer of activated B-cells (NF-κB) is able to act as a survival determinant in human mesothelial cells exposed to asbestos fibers (59). Asbestos-induced priming and activation of the NLRP3 (nucleotide-binding domain, leucine repeat containing) inflammasome initiates an autocrine feedback loop regulated via the interleukin-1 receptor in mesothelial cells, which is involved in carcinogenesis (60). Members of the extracellular signal-regulated kinases family are critical to transformation and homeostasis of human epithelioid MM (61). Hepatocyte growth factor and its receptor tyrosine kinase, c-Met, are highly expressed in most human MM cell lines (62).

BRCA1–associated protein 1 (*BAP1*) is a tumor suppressor gene located on chromosome 3p21 and a member of the ubiquitin C-terminal hydrolase subfamily of deubiquitinating enzymes that catalyzes the removal of ubiquitin from protein substrates (63). Somatic *BAP1* mutations are commonly detected in sporadic MM cases, but the reported frequency varies significantly in the literature (22% - 61% of the investigated MM specimens), probably due to methodological or ethnical dissimilarities (63).

The underlying mechanisms for the pathogenesis of MPeM are not that well-documented due to the rarity of the disease, though it is suspected that some of the afore-described pathways are similar for MPM and MPeM. Asbestos fibers can migrate through an opening in the diaphragm as well as through the lymphatic system in sufficient amounts to the peritoneum to cause MPeM, as described in heavily exposed populations (40). On the contrary, it has also been suggested that not all cases of MPeM are associated with asbestos (64). Chronic inflammation is also considered to have a role in the MPeM pathogenesis, as patients with familial Mediterranean fever have an increased risk of MPeM (40). Nonetheless, none of these hypotheses has been sufficiently documented in the literature.

1.5. GENETIC SUSCEPTIBILITY FOR MALIGNANT MESOTHELIOMA

Genetic susceptibility has long been suspected to increase the risk of MM, as only a fraction of the asbestos exposed population develop MM, while there are MM patients with no identifiable history of exposure to asbestos or asbestos-like minerals (65). The most well-investigated gene in the context of MM pathogenesis is *BAP1*. Germline *BAP1* mutations are known to induce an autosomal dominant hereditary cancer syndrome, characterized by high incidence of MM and uveal melanoma, benign atypical melanocytic lesions (MBAITs), and by an elevated risk of other malignancies, such as cutaneous melanoma, renal cell carcinoma, cholangiocarcinoma and basal cell carcinoma (66). Hereditary *BAP1* mutations were identified in two families with extraordinary high incidence of MM and in sporadic MM cases (67). In addition, it is demonstrated that patients with germline *BAP1* alterations have less aggressive disease and a 7-fold prolonged survival in comparison to patients with sporadic MM (68). The exact pathological mechanisms behind the genesis of MM and the course of the disease in *BAP1* mutation-carriers are not distinct. Recent research unveils that there are probably more cancer susceptibility genes that can predispose for MM, such as *ATM, CDKN2A, BRCA1, BRCA2, MSH6, MLH1, PALB2, TMEM127, VHL, WT1, TP53* and others (65,69–72). However, the prevalence and causative role of germline mutations in MM are not known and no standardized gene testing has been included in the guidelines for MM patients and their families yet.

1.6. CLINICAL PRESENTATION

The initial symptoms of MPM include chest pain (in 60% of the cases), dyspnea (60%) and unilateral pleural effusion, while occasionally patients have no clinical signs (2). Constitutional symptoms such as weight loss and fatigue appear later in the course of disease and are associated with a worse prognosis (2). The most common symptoms for MPeM include abdominal distension (73%), abdominal pain (40%), ascites (60%), abdominopelvic masses (93%), thrombocytosis (27%), and thromboembolic episodes (20%) (40). Palpable subcutaneous masses can present as disease progresses, especially after several surgical interventions or thoracocenteses (2). The usual sites of spread for MM are the hilar, mediastinal, internal mammary, and supraclavicular lymph nodes, together with local invasion in the pericardium, spinal cord and the contralateral lung at late disease stages (2). Distant metastases are uncommon but may occur, typically in brain and liver (73,74).

1.7. DIAGNOSIS

The diagnosis of MM is demanding due to the untypical initial symptoms, the long latency, and the challenging histopathological aspects of the disease.

1.7.1. IMAGING

Chest radiography is usually the first imaging test to detect abnormalities associated with MPM, typically unilateral pleural effusion , diffuse pleural thickening or focal pleural tumors (75). Computed Tomography (CT) is much more sensitive than chest radiography and is often an important imaging tool for the diagnosis, staging, and treatment follow-up for patients with MPM (76,77). Pleural thickening, pleura effusions, enlarged lymph nodes and pulmonary findings, such as nodular metastases or lymphangitic carcinomatosis are evident in CT-scans (78). The differentiation between benign and malign pleural thickening is crucial (79). Positron Emission Tomography (PET/CT) enhances the accuracy of staging and enables the thoracoscopic biopsy from the optimal pleural location (77,80). The majority of the MM tumors are 2-[fluorine 18]fluoro-2-deoxy-D-glucose (FDG) positive, while the FDG uptake is significantly higher in MPM than in benign lesions (Figure 1.7-1) (76,81).

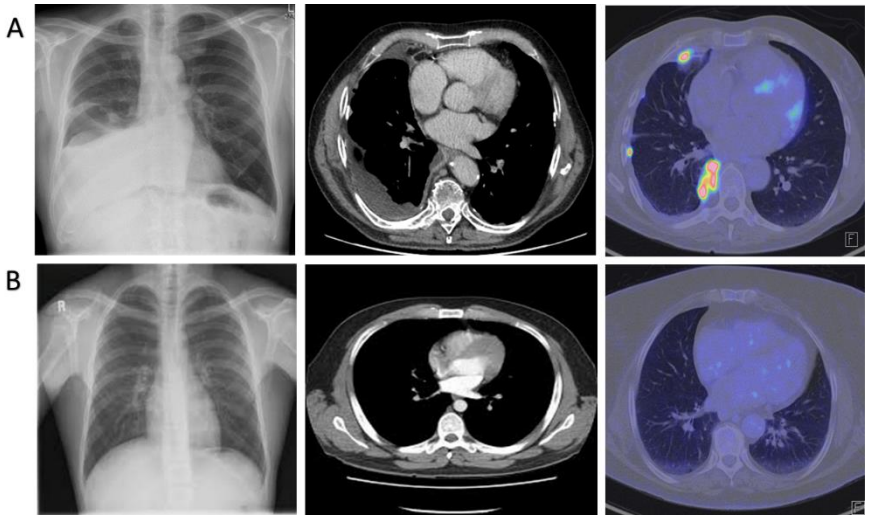


Figure 1.7-1. Representation of Chest X-ray with right pleural effusion, CT scanning with thickening of the right pleura, and PET CT scanning with increased FDG uptake in the right pleura in a patient with MPM (A) versus a normal Chest X-ray, CT scanning and PET CT scanning (B) (courtesy of Vasiliki Panou).

1.7.2. HISTOPATHOLOGY, CYTOLOGY AND IMMUNOHISTOCHEMISTRY

The gold standard for a MPM biopsy is thoracoscopy, which has a sensitivity over 90% and risk of complications <10% (82,83). When thoracoscopy is not possible, alternative examinations include CT- or ultrasound-guided biopsy with a sensitivity of 77-87% and specificity of 100% (82,83). The sensitivity of cytology for the MM diagnosis has been reported to vary from 32-76%. The most recent guidelines from the International Mesothelioma Interest Group include cytology in the diagnostic examinations of choice for MM, but in many countries histological verification is required (4,48). For MPeM, laparoscopy is the diagnostic tool of choice (84).

MM is a challenging histopathological diagnosis to set. The most frequent differential diagnosis are lung adenocarcinoma infiltrating pleura and ovarian serous carcinoma infiltrating peritoneum. Epithelioid MM may occasionally be difficult to distinguish from well differentiated MM, multicystic MM, and benign mesothelial proliferations, whereas sarcomatoid MM may be difficult to distinguish from chronic pleuritis and from other mesenchymal tumors. The key indicator of MM and other malignancies versus benign pleural conditions is the invasion of preexisting tissue, particularly adipose tissue, the presence of homozygous deletion of the 9p21 locus encoding the

p16/CDKN2A tumor suppressor gene and the immunohistochemically detectable loss of BAP1 and MTAP (85–87). The epithelioid and the sarcomatoid subtypes have different histopathological patterns, while the biphasic has both epithelioid and sarcomatoid components (4). Immunohistochemistry is essential to the MM diagnosis (4). The International Mesothelioma Interest Group recommends an immunohistochemical panel of two positive (ie, markers frequently expressed in MM) and two negative markers (ie, those that are frequently expressed in other relevant malignancies but not in MM). (4). Some broadly applied, positive MM markers for epithelioid MM are calretinin, cytokeratin 5, D2-40 (podoplanin) and Wilms tumor protein-1 (WT1) (Figure 1.7-2). Calretinin is a 29-kd calcium-binding, vitamin D–dependent protein expressed in more than 90% of epithelioid and biphasic MM, hence the most commonly used positive MM marker (4,88). Cytokeratin 5 is expressed by nearly all epithelioid and biphasic MM (89,90). False negative results can be obtained when the immunostaining for cytokeratin 5 is done in a small biopsy, but this marker has an undeniable utility in differentiating between MM and lung adenocarcinoma (4,90). Podoplanin is a mucin-type transmembrane glycoprotein strongly and selectively expressed in mesothelial cells and lymphatic endothelium but not in blood vessel endothelial cells (91). Podoplanin is particularly useful in distinguishing epithelioid MM from lung adenocarcinomas and it is also expressed in 75% of sarkomatoid MM (92,93). The Wilms' tumour (WT) gene, located on chromosome 11p13, encodes the WT1 (94). WT1 has a great utility in discriminating between epithelioid MM from adenocarcinomas or squamous cell carcinomas of the lung (95). The most important general "negative" MM markers are epithelial cell adhesion molecule (EpCAM) and Claudin 4. EpCAM, (typically detected by the antibody clones Ber-EP4 and MOC31) is widely expressed in most carcinomas while only occurring focally in 10-20% of MM cases (96). Claudin 4 has approximately the same sensitivity as EpCAM with respect to carcinomas and has not been detected in MM (97). In the differential diagnosis of MM versus lung adenocarcinoma, thyroid transcription factor and Napsin A are highly specific for lung adenocarcinoma and the sensitivity is about 80% (95,98). In the differential diagnosis of MM from serous ovarian carcinoma, PAX8 and estrogen receptor are highly specific for the latter with a sensitivity of about 90% (95,97).

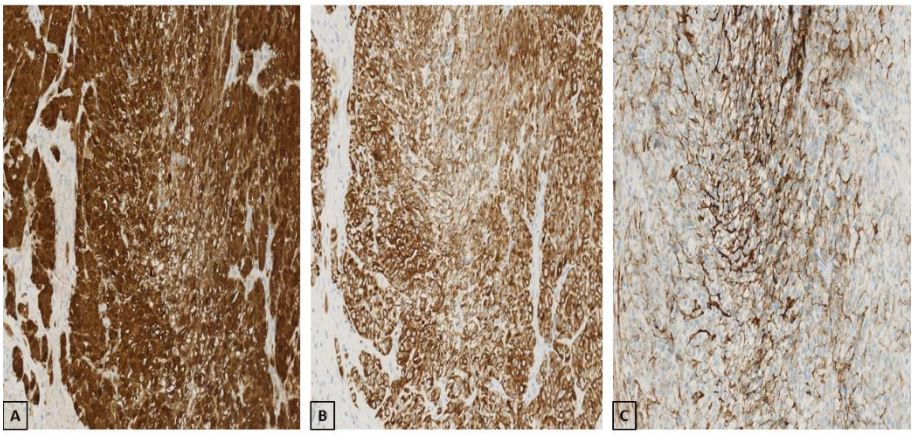


Figure 1.7-2. Immunohistochemical staining for malignant mesothelioma with (A) calretinin, (B) cytokeratin 5, (C) podoplanin (courtesy of Christos Meristoudis).

1.7.3. STAGING

The staging classification for MM is problematic, because there is no uniform system available. There have been used more than five staging systems, the latest one developed by members of the International Mesothelioma Interest Group and the Union International Contre le Cancer (99). This recent TNM-based staging system is regarded to be the most thoroughly validated of all and is recommended by European Respiratory Society and the European Society of Thoracic Surgeons for MPM. However, it is far from optimal due to its inaccuracy regarding T- and N-extent, especially based on the current imaging techniques (83). There is a consensus about the minimal pre-treatment staging assessment and tests for all MM patients that are eligible for any kind of active treatment and for combined modality regimes and surgery. Among the relevant investigations, mediastinoscopy, video-assisted thoracoscopy, endobronchial ultrasound and fine needle aspiration, FDG-PET/CT and laparoscopy are important (83).

1.8. TREATMENT

The MM has an unfavorable prognosis and a median overall survival (OS) of approximately 12-16 months for the epithelioid and 4-6 months for the sarcomatoid subtype, while less than 5% of the patients survive longer than 5 years (48,100,101).

The short survival originates from poor performance score and late stage disease at the time of diagnosis, a majority of non-resectable tumors, high perioperative mortality and morbidity, chemotherapy and radiotherapy irresponsiveness and toxicity to vital organs (48,102). It is crucial to carefully select the right patients that are eligible for the right treatment in order to achieve prolonged survival but not induce fatal side effects. Unfortunately there are limited prognostic markers for MM. Non-epithelioid subtype, pleural disease, performance status higher than 0, lactate dehydrogenase >500 IU/L, platelet count >400,000/μL, male gender and age >75 years are identified as independent predictors of poor outcome (48). MM management is more effective when a multidisciplinary team of pulmonologists, oncologists, radiologists, surgeons and pathologies assesses the patients. The typical treatment options include chemotherapy, surgery and radiation, while treatment for MPM and MPeM differ essentially (see 1.8.4).

1.8.1. CHEMOTHERAPY

Chemotherapy is most commonly administrated either for palliative purposes or as neo-adjuvant therapy prior to surgery. Since 2003, the standard of care chemotherapy for MPM is a combination therapy with cisplatin and pemetrexed with supplementation of B12 vitamin and folic acid, as it was proven to significantly increase the OS from 7-9 to 12 months in a randomized phase III study (100). The combinations of carboplatin and pemetrexed or cisplatin and gemcitabine are good alternatives for MPM patients who cannot receive cisplatin and pemetrexed, respectively (48). No single-agent chemotherapeutic and non-platinum-based polychemotherapies have shown to increase the OS for MM in the context of first-line therapy and no single-agent or polychemotherapies are proven to be efficient as second-line farmaka (83,103). A second-line option with reasonable response rate and acceptable toxicity is vinorelbine, while bevacizumab is a promising agent, as well (104–107). One of the biggest challenges for the MPM treatment is that a significant part of the patients will not respond to chemotherapy, as one of MPM characteristics is the high rate of innate and acquired chemoresistance (108).

1.8.2. SURGERY

Selected patients may be eligible for surgery with curative intent, but surgery has not been shown to increase survival in a randomized trial (109,110). The aim of curative intended surgery is to resect all visible tumor but micro-residual disease cannot be avoided and therefore, surgery is always combined with chemotherapy (48). There are two main types of surgery, extra-pleural pneumonectomy (EPP) and pleurectomy/decortication (P/D). In EPP the surgeon removes en bloc the visceral and

parietal pleurae, the lung, the ipsilateral hemidiaphragm, and the adjacent pericardium (111). P/D is defined as parietal and visceral pleurectomy without resection of the diaphragm or pericardium. There is also the extended P/D, where the diaphragm and/or pericardium are removed, and the partial pleurectomy, where there is no complete tumor resection but partial removal of parietal and/or visceral pleura for diagnosis or palliation (111). There is no international consensus on which surgical procedure is more beneficial for the MPM patients, EPP or (extended) P/D (109,111). There is complete agreement, though, that the patients that are eventual candidates for surgery require appropriate staging, cardiac and pulmonary evaluation prior to the surgical procedure. Patients with mediastinal lymph nodes, metastases, poor performance status and non-epithelioid histology are not eligible for surgery (48).

1.8.3. TREATMENT OF MALIGNANT PERITONEAL MESOTHELIOMA

Initially, MPeM patients received the same polychemotherapy with pemetrexed and cisplatin, as MPM patients, with similar or slightly better results (OS of 10 - 26.8 months) (112). Nowadays, systematic chemotherapy is reserved for patients that are not candidates for radical treatment (84). Radical treatment consists of cytoreductive surgery combined with perioperative hyperthermic intraperitoneal chemotherapy (HIPEC), and has been used for almost a decade (112). There are no standardized chemotherapeutic agents used in HIPEC (84). The chemotherapeutics are administrated at a temperature of 40.5- 43 °C in the peritoneal cavity after the end of the cytoreductive surgery, with or without early postoperative intraperitoneal chemotherapy (84). In this way, a higher concentration of cytotoxic drugs at tumor bearing sites is achieved and lower systemic side effects are observed in comparison to the systematic chemotherapy (84). The overall 5-year survival rate is reported to be between 29% – 63%, while mortality and morbidity rates vary significantly in various studies, as well (mortality rate of 0% - 20%, and morbidity rate of 8.3% - 90% with half of the studies reporting morbidity between 40% - 65%) (112). Hyperthermic intrathoraric chemotherapy has also been investigated for MPM but is of limited utility due to the risk of serious side effects (113).

1.8.4. RADIATION THERAPY

Radiation therapy can be used in the setting of palliative care for the management of chest wall pain and subcutaneous tumor spread, and as part of a multimodal approach (75,83). Prophylactic radiation after thoracocentesis does not seem to prevent subcutaneous metastases along the drainage canals and it is therefore not recommended (83). Adjuvant and neoadjuvant radiation in the context of a multimodal treatment was initially found to decrease the rate of local recurrence after

EPP (114,115). In more recent studies, hemithoracic prophylactic radiation after surgery was not associated with longer relapse-free survival, but with fatal pulmonary toxicity (116,117). Currently, radiation therapy is not a standardized part of the multimodality approach, but its use in MPM centers around the world after surgery is not uncommon.

1.8.5. EMERGING THERAPEUTIC OPTIONS

There is a number of clinical studies and trials investigating potential agents for the treatment of MM, including Epidermal growth factor receptor (EGFR) inhibitors, antibody conjugated toxins, immune checkpoint inhibitors, gene-based therapies and tumor vaccines (118). A promising future treatment could be immunotherapy. Programmed death receptor is found on the surface of T-cells and, when activated by a programmed death ligand 1 (PD-L1), it leads to cell death (119). PD-L1 is expressed in 20%-40% of MPM patients, mainly in non-epithelioid subtypes, while higher expression is correlated with worse prognosis (4.8- 5 versus 14.5-16.3 months) (120). Monoclonal antibodies against PD-L1 are being used in clinical trials for patients with locally advanced or metastatic MM, who did not respond or were unable to receive standard chemotherapy (118,121). The preliminary results show, that the agent is well-tolerated, while the majority of the patients presented partial response or stable disease and a median OS of 11.5 months (118,121,122). Photodynamic therapy, iodine-povidone (betadine) lavage and cryotherapy have also shown encouraging results but the available studies have yet to provide convincing evidence of efficacy (123–125).

1.9. BIOMARKERS FOR MALIGNANT MESOTHELIOMA

Numerous studies have attempted to identify molecular biomarkers that may have a role in diagnosis, screening, prognosis and prediction for MM but none is found to be robust enough to be adopted in clinical practice. Currently, the most promising MM biomarker is mesothelin (126,127). Mesothelin is a cell surface glycoprotein that is expressed on MM, ovarian, pancreatic, and other malignancies, while its expression on normal tissues is limited (128). It can be detected in tumor cells and in blood (128). Mesothelin can serve as a diagnostic biomarker, as it is elevated in the serum, pleural effusion and ascites of MM patients versus non-cancer population and an assay for its quantification in serum has been commercialized (MESOMARK ®) (129–131).

Other interesting biomarkers for MM are osteopontin, fibulin-3, high-mobility group box 1 (HMGB1), hyaluronic acid, micro-RNA, and proteomics (126,127). Osteopontin is an extracellular cell adhesion protein that is up-regulated in asbestos-

exposed rats and cells in vitro, while serum osteopontin levels were found to be significantly higher in patients with MPM than in a non-malignant asbestos-exposed population (132). Fibulin-3 is an extracellular glycoprotein encoded by the epidermal growth factor gene, and it is reported to be significantly elevated in plasma and pleural effusion of MPM patients versus asbestos-exposed individuals (133). Asbestos exposure induces necrosis of human mesothelial cells, and results in the release of HMGB-1 that is an inflammation mediator (134). Higher serum HMGB1 level is detected in MPM patients versus asbestos-exposed and healthy controls, suggesting HMGB1 as a potential diagnostic marker for MPM (134). Hyaluronic acid was found elevated in MM pleural effusions in the 1980s but follow-up studies were limited due to technical restrictions (135). After the development of modern assays, hyaluronic acid in pleural effusions has demonstrated similar diagnostic accuracy to mesothelin, while the accuracy improves by the combination of the two markers (136,137).

Micro-RNAs are a family of small non-coding RNAs that negatively regulate gene expression by inhibiting the translation of target messenger RNA (138). Several micro-RNAs, including micro-RNA-197-3p, micro-RNA-1281, micro-RNA-126, micro-RNA--625-3p and micro-RNA 32-3p, have been found elevated in MPM patients comparing to control groups (139–141). Micro-RNA-29 in MM was increased in serum of patients with epithelioid histology, and it was associated with more favorable prognosis (142). A group of six micro-RNAs has been documented to be of prognostic value for MPM patients, who have undergone EPP (143). Slow Off-Rate Modified Aptamers (SOMAmers) are short, single stranded deoxynucleotides with the ability to function as capture reagents (144). By the use of SOMAmer technology, a candidate 13 biomarker panel, consisting of novel inflammatory and proliferative proteins, was developed for the detection of MPM in asbestos-exposed individuals (144). Its sensitivity ranged from 77% to 96% depending on the disease stage (sensitivity of 77% for stage I, 93% for stage II and 96% of stage III-IV) (144).

1.10. ASBESTOS AND MALIGANT MESOTHELIOMA IN THE REGION OF NORTH DENMARK

The Region of North Denmark has had a high incidence of MPM compared to the rest of Denmark for more than three decades (data from the NORDCAN database). The crude and age-standardized rate of MPM for the North Denmark Region has been higher since 1980 and almost double as high since the late 1990´s compared to the rest of Denmark (Figure 1.9-1, Appendix A). The highest age-standardized rates were observed in 2011 (7.3/100,000), 1999 (7.2/100,000), 2006 (7.0/100,000), 2015 (6.6/100,000) and 2010 (6.5/100,000), but the top is yet to be reached. Data about the incidence of MPeM are not available.

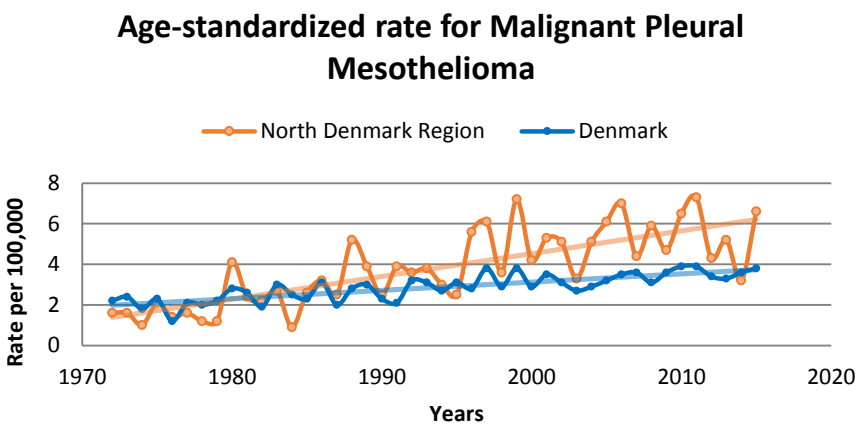


Figure 1-9.1. Representation of the age-standardized rate for Malignant Pleural Mesothelioma for the Region of North Denmark and Denmark during 1972-2015 (based on data from NORDCAN, http://www-dep.iarc.fr/NORDCAN/English/frame.asp).

The high incidence is associated to the asbestos industry that has operated in the area. The only Danish Asbestos Cement Factory (DAF) was located centrally in the city of Aalborg, the Region's capital, neighboring with numerous residencies and four schools. DAF was founded in September 1927, while the manufacture of its principal product, asbestos cement sheeting, was initiated in April 1928 (historical data from Aalborg Archive). Chrysotile asbestos was mainly used, with the exception of the period 1946-1968, where small amounts of amosite (10%) and crocidolite (1%) were also utilized (145). From 1928-1933 asbestos was imported from Russia, Canada and Rhodesia but since 1935, the company's primary supplier was the Amiandos mine located at the mountain Troodos in Cyprus (historical data from Aalborg Archive). The asbestos import varied throughout the years, it started from 17,000-25,000 kg in 1928 and peaked in the 1970s with approximately 25,000-34,000 metric tons annually ((146), historical data from Aalborg Archive). The Danish asbestos import was quite high in comparison to the Scandinavian countries, e.g. higher compared to Norway and Sweden, and this is reflected in the high MM incidence, especially in the Region of North Denmark (Figure 1-9.2, (147)). A total of 8,000 men and 590 women were working at DAF before asbestos was banned (146).

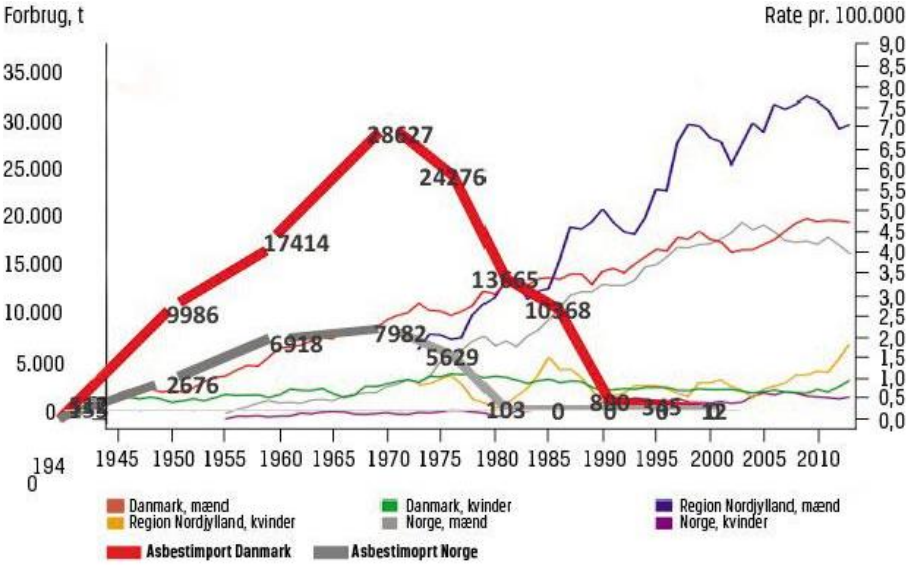


Figure 1-9.2. Representation of the use of asbestos (forbrug) in tons (t) during 1940-2000 and the incidence of Malignant Mesothelioma (MM) in Denmark and Norway. Orange line: MM incidence for men in Denmark, Green line: MM incidence for women in Denmark, Blue line: MM incidence for men in the Region of North Denmark, Yellow line: MM incidence for women in the Region of North Denmark, Grey line: MM incidence for men in Norway, Purple line: MM incidence for women in Norway, Red bold line: Asbestos import in Denmark, Grey bold line: Asbestos import in Norway. Reproduced from Ugeskr Læger 2018;180:V02180128 with permission from the publisher and Oluf D. Røe.

Asbestos was officially banned in Denmark in 1980 but dispensation was given for manufacturing of brake blocks and asbestos cement products, which was the principal product of DAF, thus the production line was not affected initially (historical data from Aalborg Archive). In 1986 a stricter regulation was voted by the parliament, banning construction of all kind of asbestos-containing products and only allowing the use of asbestos in brake blocks, which was further prohibited in 1988 (historical data from Aalborg Archive). The Amiantos mine was donated in 1986 to the Bishop of Limassol and asbestos mining was terminated in 1988 (historical data from Aalborg Archive). A large shipyard was also based in Aalborg, which was established in 1912 and closed in 1988. There is no consistent information about the exact total number of employees at Aalborg Shipyard during 1912-1988, but it is estimated to be between 15,000-25,000 individuals (Figure 1-9-3). Another big shipyard operated in the city of Frederikshavn from 1870-1999 (historical data from Aalborg Archive). Crocidolite asbestos was mainly used in both shipyards.

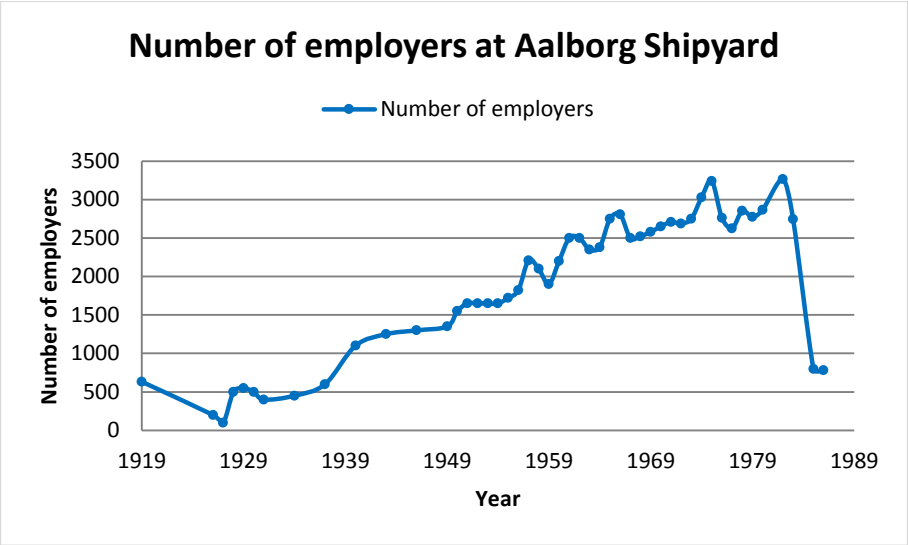


Figure 1-9.3. Representation of the approximate number of employees at Aalborg Shipyard during 1912-1988 (data from Aalborg Archive and from the book ''Byens Værft'' by Flemming Nielsen.

As a result, an extensive number of the residents of the Region of North Denmark, and particularly of Aalborg, have been exposed to asbestos through either their job, or the environment or their household members that were asbestos workers. The studies about asbestos exposure and its impact on the affected population in Aalborg and the Region of North Denmark are scarce. One study from 1989, investigated the incidence of cancer and mortality among 8580 male and female workers at DAF between 1928 and 1984 and showed increased overall mortality, cancer mortality and overall incidence of cancer in men, compared to the Danish male population; the equivalent was not seen in women (146). A subsequent study by the same author examined the histological patterns of the lung cancer cases for this population, confirming a link between lung adenocarcinoma and asbestos (145). A recent study explored the risk of MM in children who attended a school near DAF and concluded that they had a significantly higher risk of MM as adults (148). No large studies have been conducted previously to investigate the effect of occupational and non-occupational asbestos exposure on the total population of North Denmark and its causal relationship with MM.

To summarize, the high historical asbestos burden of Aalborg culminated in a particularly high and increasing MM incidence for the Region of North Denmark. This resulted in hundreds of male and female patients diagnosed with MPM and MPeM through the last decades, a man-made epidemic. For all these patients, there are available data, with clinical, pathological and asbestos exposure information through the medical records and the high-quality Danish registries. Thus, through

these unique and validated repositories, large studies on male and female populations with MM can be performed.

CHAPTER 2. AIMS OF THE THESIS

As illustrated in Chapter 1, there is a plethora of studies examining biomarkers for the diagnosis, prognosis and prediction of MM but their clinical utility is highly limited. Furthermore, non-occupational asbestos exposure and genetic susceptibility are two under-investigated subjects in the context of MM carcinogenesis, mostly due to the sparse studies on large populations, consisting of both men and women. Therefore, this thesis hypothesizes that:

i. The reports about the sensitivity and specificity of the established and emerging diagnostic, prognostic and predictive MPM biomarkers differ to a large extent and this hinders their clinical utility.
ii. Non-occupational asbestos exposure has a significant role in the tumorigenesis of MM.
iii. The asbestos exposure profiles for men and women with MM present with considerable dissimilarities.
iv. The asbestos exposure pattern and the patient´s gender can influence the location of the MM development and the histopathological MM subtype.
v. Inherited mutations other than in the *BAP1* gene are implicated in the MM genesis and germline mutation carriers share common clinical characteristics.

The study aims are to:

1. Summarize the most important current and most promising future biomarkers within the diagnosis, prognosis and prediction for MPM.
2. Explore the extent and impact of non-occupational asbestos exposure for women and men with MM.
3. Elucidate and compare the asbestos exposure patterns for the male and the female MM patients.
4. Examine whether the histopathological MM subtype (epithelioid or non-epithelioid) and the MM location (pleura or peritoneum) are associated with the type of asbestos exposure or the gender of the patient.
5. Assess the prevalence and the spectrum of germline mutations in MM.
6. Determine disease characteristics that can predict the presence of a germline mutation.
7. Explore genetic pathways in MM carcinogenesis.

Three studies were planned and performed in order to investigate the above-mentioned aims, a literature review, a retrospective, observational study and a prospective study (Figure 2-1.1). The literature review (Study I) discusses established and emerging MPM biomarkers with current or potential clinical impact in diagnosis, prognosis and prediction (Aim 1). The retrospective, observational study (Study II)

explores the role of non-occupational asbestos exposure in women (Study IIa) and men (Study IIb) with MM (Aims 2, 3 and 4). The prospective study (Study III) examines the prevalence, spectrum and clinical predictors of germline mutations in cancer susceptibility genes in MM (Aims 5, 6 and 7). The four papers will from heron be referred to as named above.

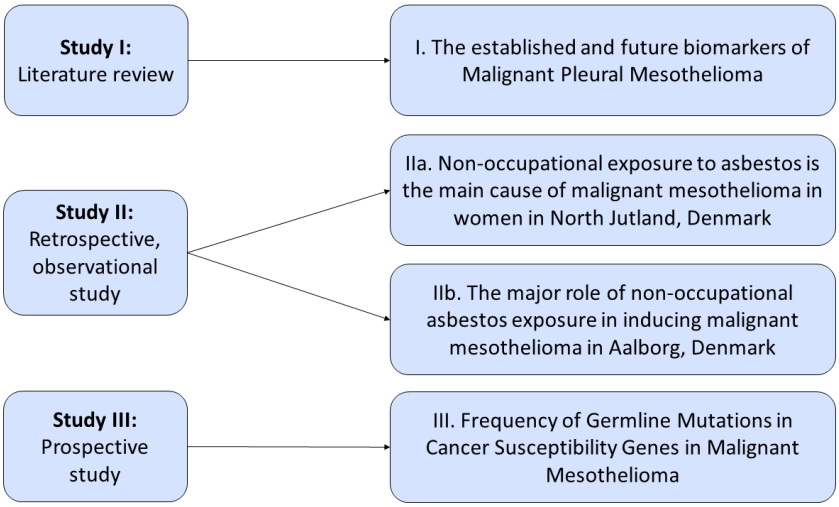


Figure 2-1.1. Overview of studies included in this thesis and titles of corresponding papers.

CHAPTER 3. PRESENTATION OF THE STUDIES

The principal methods and main findings of the Studies I, II and III will be outlined in this chapter. For further details, please refer to the manuscripts.

3.1. STUDY I

3.1.1. MATERIALS AND METHODS

A literature research without a lower data limit was conducted on the databases PubMed and PLOS ONE by using the following keywords: "malignant mesothelioma" and "biomarkers", "immunohistochemistry", "BAP-1", "deformability cytometry", ''fibulin-3", "genome profile", ''hyaluronan'', "long non-coding RNA", "mesothelin", "microRNA", "osteopontin", "proteomics", "soluble mesothelin related protein''. The reference lists of relevant publications were utilized, as well. Articles written in English and published until April 26th, 2015 were reviewed.

3.1.2. RESULTS

The search revealed contradicting studies about diagnostic, prognostic and predictive biomarkers for MM. Markers that are currently in clinical use or present with a potential for MM are presented in this review. Immunohistochemistry is the cornerstone of the MPM diagnosis. There are no single immunohistochemical markers that are sensitive and specific enough to set the diagnosis, especially for sarkomatoid MM. Thus, a panel of two immunohistopositive and two immunohistonegative markers has been included in the guidelines for the MPM diagnosis since 2009. Calretinin, cytokeratin 5, podoplanin and WT1 are among the most important immunohistopositive diagnostic markers for MM. Carcinoembryonic antigen, Claudin-4, epithelial cell adhesion molecule, thyroid transcription factor estrogen receptor and mammaglobin are some of the most useful immunohistochemical MM markers (Table 3-1.1). Mesothelin in serum and pleural fluid is the most promising diagnostic marker for MM and the only approved marker for the monitoring of non-sarcomatoid MPM by Food and Drug Administration. Osteopontin, hyaluronate, fibulin-3, deformability cytometry, selected reaction monitoring assay technology,

fluorescence in situ hybridization assay and microRNA present with a certain interest among the MM biomarkers in serum, plasma and pleural fluid, as well (Table 3-1.2). Long non-coding RNA, proteomics and gene expression profiling are emerging biomarkers for the MM diagnosis and prognosis, while BAP1 is the most robust MM susceptibility marker (Table 3-1.2). There are no validated predictive MPM biomarkers yet, but in studies, thymidylate synthase, excision repair cross-complementation group 1 and BAP1 have shown some potential in predicting survival and response after pemetrexed and vinorelbine-cisplatin chemotherapy.

Tumor	Mesothelioma markers				Broad spectrum carcinoma markers			Lung ADCA	Breast ADCA	
	CR	CK5	PDP	WT 1	CE A	CL 4	EpCA M	TTF1	ER	M G
MM epitheli	+	+/-	+	+/-	-(+)f	-	-/+f	-	-	-
Lung ADCA	-/+f	-/+f	- (+)f	-	+/-	+	+	+/-	-/+	- (+)f
Breast ADCA	-/+b	-/+b	-/+	-/+	+/-	+	+	-(+)f	+/-	+/-

+ : >90% positive
+/- : 50-90% positive
-/+ : 10-<50% positive
-(+) : 1-<10% positive
- : <1% positive
f: focal when positive
b: basal-like type in most cases when positive
ADCA: adenocarcinoma, CEA: carcinoembryonic antigen, CK5: cytokeratin 5, CL4: Claudin-4, CR: calretinin, EpCAM: epithelial cell adhesion molecule, ER: estrogen receptor alpha, MG: Mammaglobin, MM: malignant mesothelioma, PDP: podoplanin, TTF1: thyroid transcription factor-1, WT1: Wilms' tumour-1 (nuclear reaction)
Table 3-1.1. Commonly used markers and their expression pattern in the immunohistochemical classification of epithelioid malignant mesothelioma versus lung and breast adenocarcinoma.

Biomarker	Location	Sensitivity	Specificity
Mesothelin/SMRP	Serum, pleural effusion	68 - 90%	80 - 95%
Osteopontin	Serum, plasma, body fluids, tissue	58 - 95%	53 - 95%
Fibulin-3	Plasma, pleural effusion	22 - 94%	71 - 100%
Hyaluronate	Pleural effusion, serum	50 - 56%	98 - 100%
MicroRNA	Tissue, plasma	63 - 100%	74 - 95%
SOMAmers	Plasma	77 - 96%	91 - 95%
lncRNA	Tissue	71%	100%
Gene expression ratio test	Tissue	100%	90%

lncRNA: long non-coding RNA
SOMAmers: Slow Off-rate Modified Aptamers
SMRP: Serum Mesothelin Related Protein

Table 3-1.2. Selection of potential diagnostic mesothelioma biomarkers, their location, sensitivity and specificity, as reported in several studies.

3.1.3. CONCLUSION

This study managed to outline the established and future diagnostic, prognostic and predictive biomarkers for MM (Aim 1). The gold standard of MM diagnosis is immunohistochemistry. Due to the difficulty of establishing the diagnosis, the International Mesothelioma Interest Group introduced guidelines with two positive and two negative markers to increase sensitivity and specificity. Serum and pleural fluid mesothelin is the only approved circulating biomarker for diagnosis and monitoring of treatment. New markers are constantly emerging, such as BAP1.

3.2. STUDY II

3.2.1. MATERIALS AND METHODS

3.2.1.1 Study Population

Histological and cytological samples from MPM and MPeM that were stored in the archives of the Institute of Pathology during 1970-2015 were considered for inclusion in the study. All the samples were reclassified by two experienced pathologists individually to verify the diagnosis. The reclassification was based on a 5-tiered scheme with the following categories: 1. Definitely MM, 2. Probably MM, 3. Likely MM, 4. Unlikely MM and 5. Definitely not MM. Additional immunostainings according to the International Mesothelioma Interest Group guidelines were applied, when necessary. Biopsies classified as 'definitely', 'probably' and 'likely' MM were included at the study. All the included patients had an additional clinical diagnosis of MPM or MPeM, and if this was not the case, they were excluded from the study.

3.2.1.2 Data Sources and Recovery

Information about asbestos exposure, MM characteristics and patient survival were acquired from a plethora of sources. The Danish Supplementary Pension Fund Registry, the Danish Civil Registration System and the patients' medical records (assessments from lung specialists and occupational health specialists in particular) provided information about asbestos exposure. The MM subtype, epithelioid or non-epithelioid (including sarcomatoid and biphasic) and the MM location, pleura or peritoneum, were registered through the archives of the Institute of Pathology and the Danish Cancer Registry. A unique personal identification number allowed us to combine data from all the above registries for each patient. The Nordic Cancer database, NORDCAN and Statistics Denmark were also used.

3.2.1.3 Asbestos Exposure

Potential asbestos exposure was categorized in three main groups, occupational, non-occupational and unknown exposure, and combinations of those (Figure 3-2.1). Occupational asbestos exposure characterized the asbestos workers. Non-occupational asbestos exposure included domestic exposure, for patients sharing residence with asbestos workers and environmental exposure, for people living or working within 10km of an asbestos plant. The choice of the 10km radius was based on previous studies. All patients that had worked with asbestos were placed in the 'Occupational exposure' group, regardless if they were also exposed to asbestos non-occupationally. Patients with domestic and/or environmental exposure were allocated

to the 'Non-occupational exposure' group. The 'Unknown exposure' category was used for the cases, where no asbestos exposure could be identified.

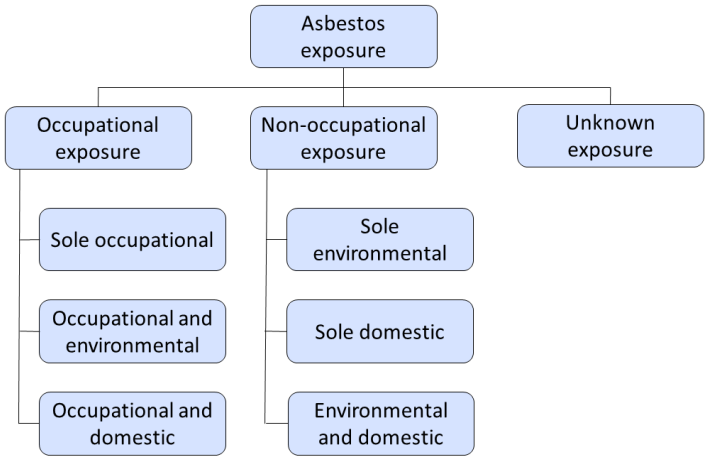


Figure 3-2.1. Categorisation of asbestos exposure.

3.2.1.4 Statistical Analysis

The population demographics were characterized by descriptive statistics. Categorical data were presented as total number (frequency/%) and continuous data as either mean (standard deviation (SD)) or median (interquartile range (IQR)) depending on normal distribution, evaluated by the Shapiro-Wilk test. Two-sided Fisher´s exact and Pearson's chi-square test were used to test differences between two groups of categorical variables, and logistic regression to correlate a dependent with two independent categorical variables. The independent t-test or the Wilcoxon signed-rank test was applied to test the means of normally or not-normally distributed groups, respectively. Statistical significance was reached when $p < 0.05$.

Study IIa

For the study IIa, the cumulative incidence and the spatial relative risk (RR) of MM among women that resided the parishes of the North Denmark region during 1974–2015 was determined. The median number of female residents for every five-year period during 1980-2015 was registered in order to assess the number of women at risk in each parish and in Denmark. Henceforth, the ratio of the number of the cumulated MM cases and the estimated number of female residents at risk, divided by 41 years of observation was used to calculate the cumulative incidence of MM per 100,000 residents in the period 1974–2015 for each parish and Denmark. By dividing

the cumulative incidence of the parish with the total cumulative incidence of Denmark, the RR over the period 1974–2015 for each parish was obtained.

Study IIb

For the study IIb, the cumulative incidence of occupational and non-occupational MM cases during 1970-2015 for the male residents of the parishes of the Region of North Denmark was determined. An estimation of the number of men at risk in each parish was calculated using the median number of male residents for every five-year period during 1980-2015. The cumulative incidence of MM was computed by the ratio of the number of cumulative MM and the estimate of men at risk divided by 46 years of observation per 100,000 residents. The cumulative MM incidence for all the MM cases of each parish was divided with the cumulative incidence of Denmark in order to find the RR of MM for the male residents of the North Denmark Region compared to the Danish men.

For both Study IIa and Study IIb, the 95% Confidence Intervals (CI) were calculated by the Clopper-Pearson's method. No age adjustment was made due to lacking information about the age distribution in the parishes and in Denmark.

3.2.2. RESULTS

3.2.2.1 Population Demographics

Out of 575 patients with MPM and MPeM from the archives of the Institute of Pathology, Aalborg University Hospital, 427 were included in the study, hereof 91 women and 336 men (male:female ratio of 3.7:1) (Figure 3-2.2). The population characteristics are summarized in Table 3-2.1.

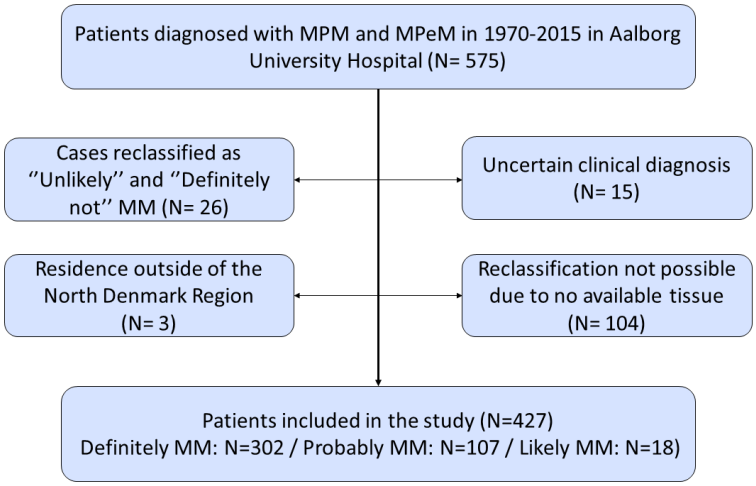


Figure 3-2.2. Inclusion flowchart for Study II.

Total number of patients, N (%)	**427 (100%)**
Gender, N (%)	
Men	336 (79%)
Women	91 (21%)
Disease topography, N (%)	
Pleura	382 (90%)
Peritoneum	45 (10%)
Disease subtype, N (%)	
Epithelioid	260 (61%)
Non-epithelioid	144 (34%)
Unknown	23 (5%)
Age at diagnosis, mean (SD)	67.6 (10.7)
Age at first exposure, median (IQR)	1 (22)
Exposure duration- environmental, median (IQR)	55 (17)
Exposure duration- occupational, median (IQR)	23 (22)
Exposure latency, median (IQR)	60 (28)

Table 3-2.1. Patient demographics.

3.2.2.2 Malignant Mesothelioma in the North Denmark Region

The crude incidence of MM for the men and women of the North Denmark Region is particularly high and still increasing (Figure 3-2.3). Especially the women of the Region of North Denmark have 1.9-2.6 higher risk of developing MPM in comparison to the women of the other Danish regions (Table 3-2.2).

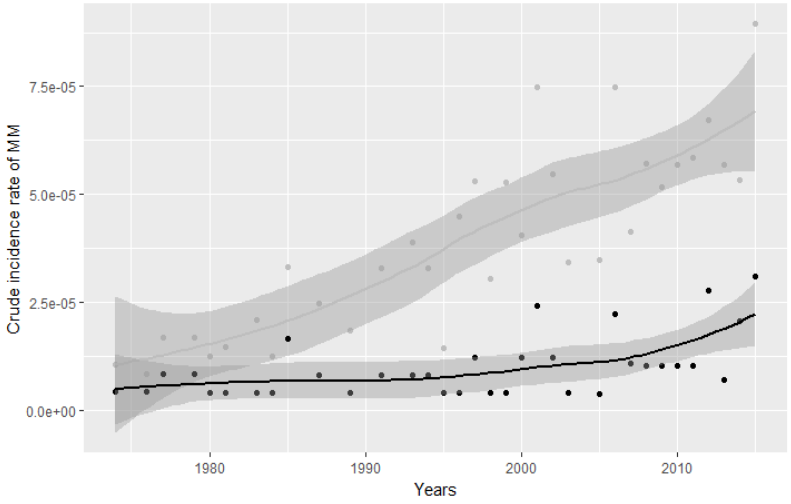


Figure 3-2.3. Crude incidence rate for malignant mesothelioma (MM) for the women (black color) and both men and women (grey color) of the Region of North Denmark, Denmark.

Danish Regions	Crude rate	Relative risk ratio
North Denmark	1.3	-
Central Denmark	0.7	1.9
South Denmark	0.7	1.9
Capital	0.6	2.2
Zealand	0.5	2.6

Table 3-2.2. Malignant mesothelioma incidence per 100,000 inhabitants in Danish regions and relative risk ratio as to the Region of North Denmark during 2010-2014. Data from Nordcan and the Danish Cancer Registry.

Study IIa

A map over the Region of North Denmark demonstrated a MM ´´hotspot´´ for women, consisting of 20 parishes with shared borders in the city area of Aalborg and within 10km from asbestos plants (Figure 3-2.4). In these parishes, the MM cumulative incidence ranged 0.72–7.21/100,000 person-years versus 0.69/100,000 person-years for Denmark. Women residing in these parishes had also higher RR of MM than the Danish women. Particularly the parish where DAF was located had the highest incidence of 7.21 per 100,000 person-years and the highest RR for MM (RR=10.5 for all the patients, RR=2.9 for the environmentally exposed) compared to the rest of Denmark (Figure 3-2.4, Appendix B).

Study IIb

Study IIb revealed a similar ´´hotspot´´ of 16 continuous parishes within a 10km radius from asbestos industries for the male population. Inside this ´´hotspot´´, the cumulative incidence per 100,000 person-years for MM was higher than the other parishes of the North Denmark Region, for both the occupationally and non-occupationally exposed male MM patients (Figure 3-2.5, Appendix B). When all exposure types were taken into consideration, the male residents of ten parishes inside this ´´hotspot´´ had higher RR of MM compared to the Danish men (Table 3-2.3). The highest cumulative incidence and RR of all was recorded in the parish, where Aalborg shipyard was located, whereas the parish where DAF operated, presented with the fourth highest RR (Table 3-2.3).

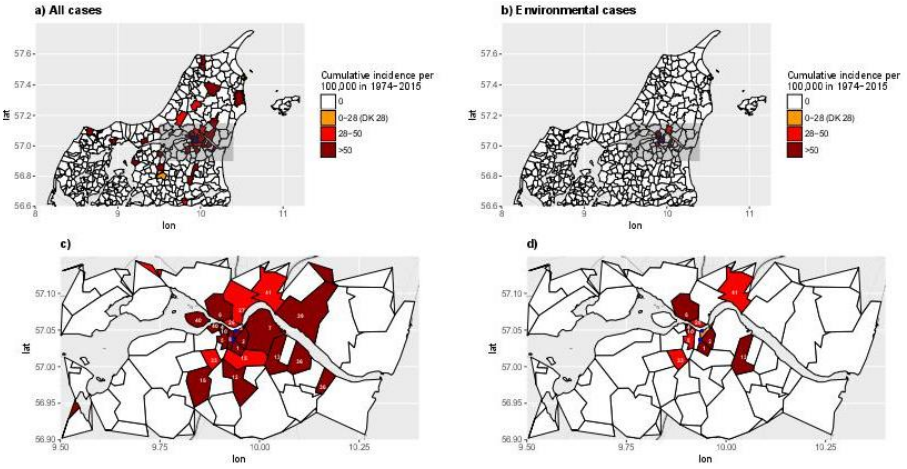


Figure 3-2.4. Malignant mesothelioma incidence for women in the parishes of the Region of North Denmark (a, b) and the city of Aalborg (c, d). Further information about the parishes shown in figures 3-2.4a/4c and 3-2.4b/4d can be found in Tables 1a and 1b in the Appendix B, respectively. The Aalborg shipyard (upper) and the Danish asbestos cement factory (lower) are illustrated as blue triangles. White areas on the map have no MM female cases in 1974–2015.

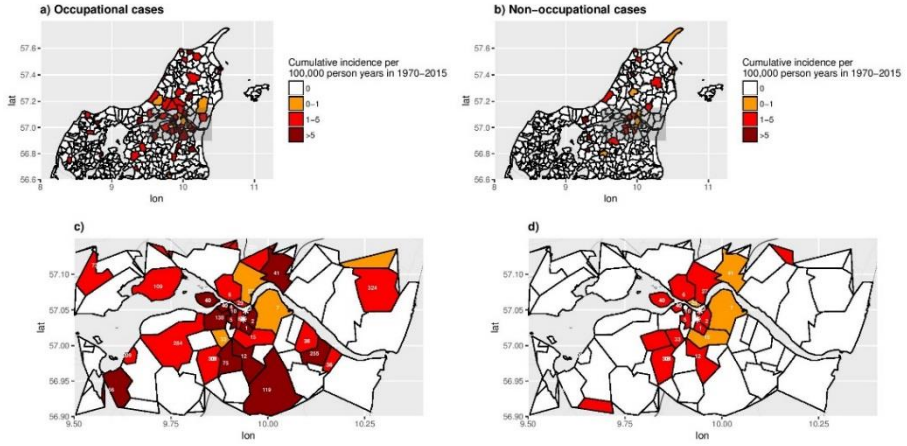


Figure 3-2.5. The cumulative incidence per 100,000 person years for malignant mesothelioma for men in the parishes of the Region of North Denmark (a, b) and the city of Aalborg (c, d). Further information about the parishes shown in figures 3-2.5a/5c and 3-2.5b/5d can be found in Tables 2a and 2b in the Appendix B, respectively. The Aalborg shipyard (upper) and the Danish asbestos cement factory (lower) are illustrated as white stars. White areas on the map have no MM male cases in 1970–2015.

Parish name	Parish number	Number of MM cases	Cumulative incidence per 100,000 person- years	RR (Parish / DK*)	95% CI
Vejgaard	2	29	10.02	4.09	2.84, 5.89
Sankt Markus	3	25	15.06	6.14	4.15, 9.10
Hans Egedes	1	13	7.87	3.21	1.87, 5.54
Ansgars	5	13	8.87	3.62	2.10, 6.23
Lindholm	6	11	5.60	2.28	1.26, 4.12
Budolfi	10	10	7.32	2.98	1.61, 5.55
Noevling	12	9	11.45	4.67	2.43, 8.97
Vesterkaer	40	9	11.26	4.59	2.39, 8.82
Vodskov	41	7	6.57	2.68	1.28, 5.62
Svenstrup	301	7	6.14	2.51	1.19, 5.26

* Denmark (DK) has a cumulative incidence of male MM of 2.45 per 100,000 person years.

Table 3-2.3. Representation of the cumulative incidence and relative risk of MM for the male residents of parishes in the North Denmark Region (all types of exposure are taken into consideration). DAF was located in parish number 2 and Aalborg shipyard in parish number 3.

3.2.2.3 Asbestos Exposure Patterns and Disease Characteristics

Study IIa

The main sources of asbestos exposure for the women were environmental (N=21, 22%), domestic (N=9, 10%) and combination of those (N=31, 34%) (Figure 3-2.6). The domestic exposure occurred through their husbands (N=26), fathers (N=7), sons (N=4) or both husbands and sons (N=3), who were asbestos workers (Table 3-2.4). The women with non-occupational exposure to asbestos tended to develop MPM (N=54) rather than MPeM (N=7), whereas occupationally exposed developed MPeM (N=3) rather than MPM (N=5) (p=0.046) (Figure 3-2.6).

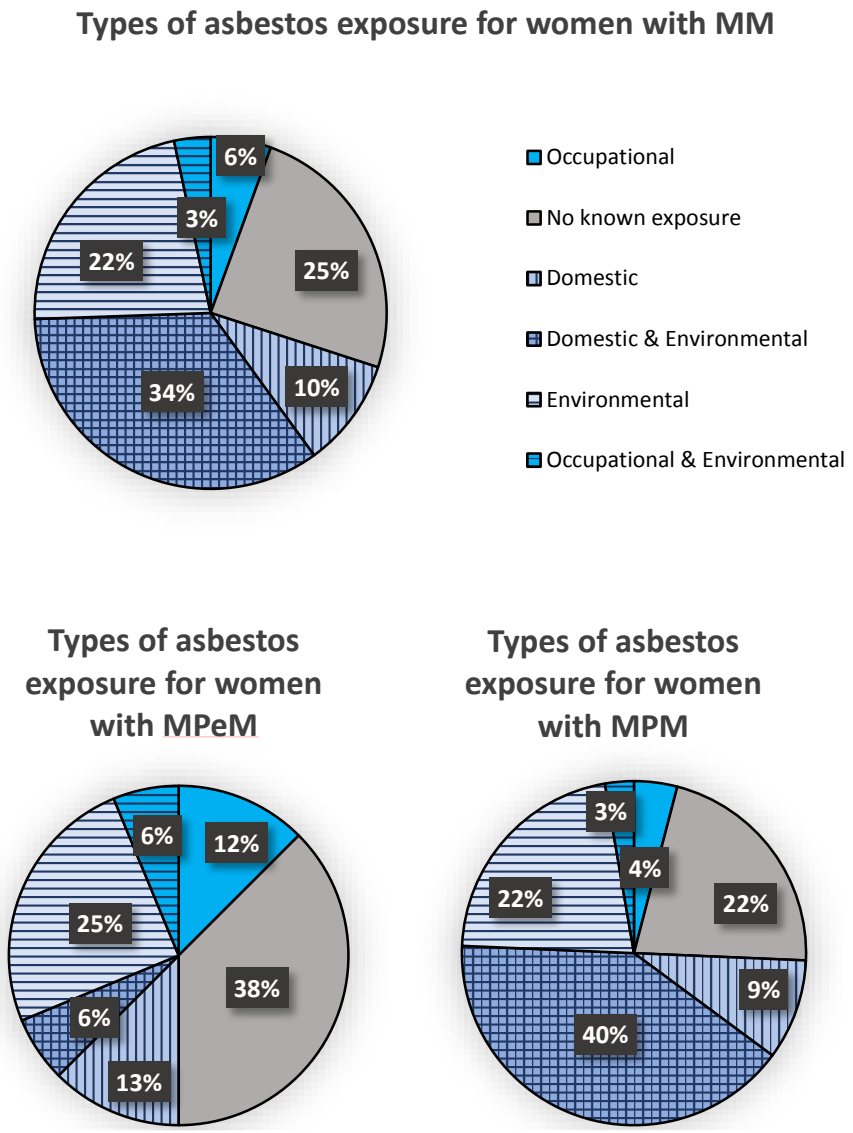


Figure 3-2.6. Types of asbestos exposure for the 91 women MM, then further categorized in cases with MPM and MPeM.

Number of cases	Relatives workplace or employment type
11	Aalborg Shipyard
11	DAF
8	Construction worker
2	Worker at pipe factory
2	Insulator
2	Electrician
1	Car mechanic
1	Engineer
1	DAF and Aalborg Shipyard
1	Worker installing asbestos roof

Table 3-2.4. Employment information for the relatives of the female MM patients. The data resulted from assessment by an occupational health expert and from data from the Danish Supplementary Pension Fund Registry and the Danish Civil Registration System.

Study IIb

In this study, 66% (N=282, male:female ratio of 34:1) of the patients had occupational and 25% (N=105, male:female ratio of 1:1.4) non-occupational asbestos exposure, including 60 cases (14%, male:female ratio of 1.9:1) with pure environmental exposure. The men and the women had significantly different exposure profiles ($p<0.0001$ for all groups) (Figure 3-2.7). Most of the men had a combined occupational and environmental exposure (N=191, 57%) or were exposed to asbestos through their jobs (N=82, 25%) or environment (N=39, 12%). The most popular work places for the occupationally exposed patients were the shipyards (N=113, 40%), construction industry (N=44, 15%) and DAF (N=39, 14%) (Table 3-2.5). MPeM was more prevalent among women than men ($p=0.016$, Odds ratio (OR)=4.34, 95%CI=[1.31,14.35]). Individuals with occupational asbestos exposure were prone to develop non-epitheliod MM, whereas the epithelioid subtype was more frequent in the non-occupationally exposed patients ($p=0.008$, OR=0.38, 95%CI=[0.186, 0.777]) (Figure 3-2.8).

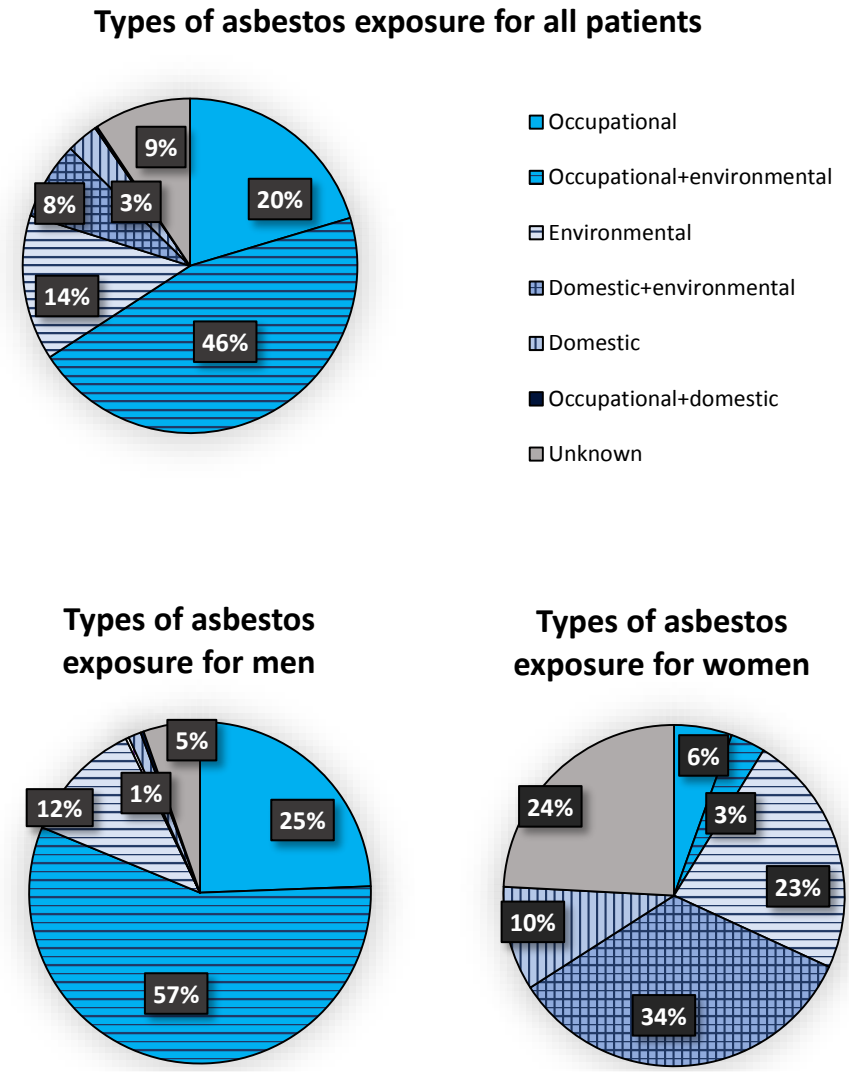
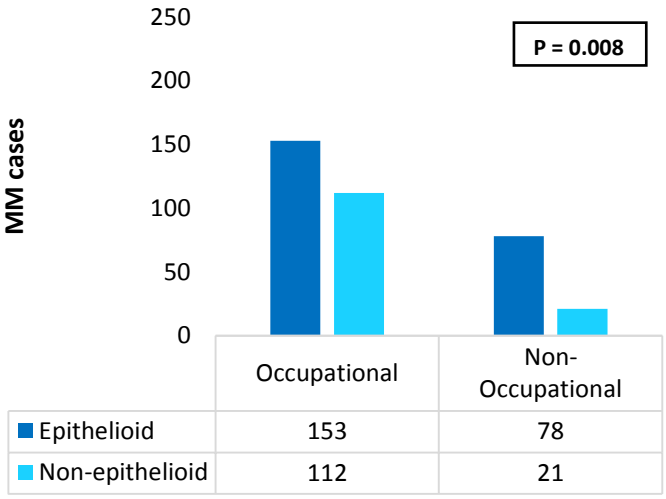


Figure 3-2.7. Types of asbestos exposure for the total, the male and the female population.

A



B

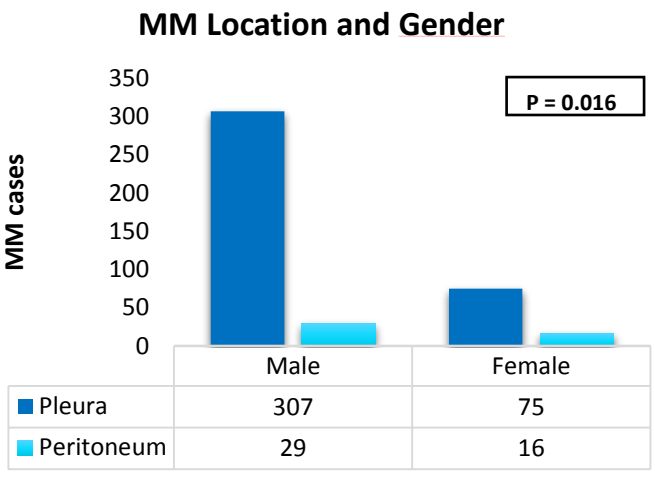


Figure 3-2.8. A. The subtypes of MM for the occupationally and non-occupationally exposed patients. The occupationally exposed patients had an overrepresentation of non-epithelioid, while the non-occupationally exposed patients of epithelioid MM (p= 0.008, OR= 0.38, 95%CI= [0.186, 0.777]). B. The location of MM for men and women. A larger proportion of women develop MPeM in comparison to men (0.016, OR= 4.34, 95%CI= [1.31,14.35]).

Profession/Industry	Men, N (%)	Women, N (%)
Worker at shipyard	112 (40.9%)	1 (12.5%)
Construction industry	44 (16.1%)	0
Worker at DAF	36 (13.1%)	3 (37.5%)
Unskilled laborer	29 (10.6%)	0
Other	13 (4.7%)	3 (37.5%)
Worker at DAF and shipyard	14 (5.1%)	0
Smith	12 (4.4%)	0
Electrician	7 (2.6%)	0
Mechanic	6 (2.2%)	0
Unknown	1 (0.4%)	1 (12.5%)

Table 3-2.5. Professions of male and female MM patients that were occupationally exposed to asbestos. The asbestos exposure information resulted from assessment by an occupational health expert and from data from the Danish Supplementary Pension Fund Registry.

3.2.3. CONCLUSION

Study IIa showed that non-occupational asbestos exposure is the main cause of MM for the women in the Region of North Denmark. Furthermore, it identified a high-incidence and high-risk ´´hotspot´´ for the exposed population within 10km from asbestos industries in the city of Aalborg (Aim 2). Lastly, the study indicated that occupational asbestos exposure was linked to MPeM and non-occupational asbestos exposure was linked to MPM in women (Aim 4).

Study IIb demonstrated that non-occupational asbestos exposure was implicated in the MM pathogenesis for the majority of the men (Aim 2). A similar ´´hotspot´´ with the one in Study IIa was defined for the male population, where the cumulative incidence for MM was increased compared to the North Denmark Region. Men residing in ten parishes inside this ´´hotspot´´ had higher RR of MM than the Danish men. The male and female patients were found to have profoundly different asbestos exposure profiles, but combined asbestos exposures was common for the total population (Aim 3). An overrepresentation of MPeM among women compared to men was noticed, while non-occupational asbestos exposure was associated to epithelioid and occupational exposure to non-epithelioid MM subtype (Aim 4).

3.3. STUDY III

3.3.1. MATERIALS AND METHODS

3.3.1.1 Study population

The study population consisted of MM patients treated at the University of Chicago MM clinic during April 2016-August 2017 and of deceased patients who had previously consented to a tumor-bank protocol. Two trained interviewers used a standardized questionnaire to obtain detailed information about personal and family history of cancer and asbestos exposure from all the patients. Asbestos exposure was grouped as primary for occupationally exposed individuals and secondary, for domestic and environmental exposure. Further clinical information was acquired from the medical records.

3.3.1.2 Germline Mutations

A targeted gene panel, designed by The University of Chicago Genetic Services Laboratory was used on saliva or peripheral blood from the included patients to sequence 85 cancer susceptibility genes in order to identify pathogenic and likely pathogenic germline variants (Table 3-3.1). Nonsense, frameshift, splice site, and missense variants with known damaging effect on protein function, in genes with known moderate-to-high penetrance cancer susceptibility were included in the study. Sanger sequencing validated all the genetic findings, and they were correlated with the clinical information and family history, as well. The frequency of germline mutations for a non-cancer population was assessed through the Exome Aggregation Consortium (ExAC). All genetic analyses were performed according to the American College of Medical Genetics guidelines.

ANKRD26	*APC*	*ATM*	*BAP1*	*BARD1*	*BLM*
BMPR1A	*BRAF*	*BRCA1*	*BRCA2*	*BRIP1*	*CDH1*
CDK4	*CDKN1B*	*CDKN2A*	*CEBPA*	*CHEK2*	*CTNNB1*
DKC1	*EPCAM*	*FANCA*	*FANCB*	*FANCC*	*FANCD2*
FANCE	*FANCF*	*FANCG*	*FANCI*	*FANCL*	*FANCM*
FH	*FLCN*	*GATA2*	*GREM1*	*HRAS*	*KIT*
KRAS	*MAX*	*MEN1*	*MET*	*MITF*	*MLH1*
MLH3	*MRE11A*	*MSH2*	*MSH3*	*MSH6*	*MUTYH*
NBN	*NF1*	*NF2*	*PALB2*	*PAX5*	*PDGFRA*
PIK3CA	*PMS1*	*PMS2*	*POLD1*	*POLE*	*PTCH1*
PTEN	*PTPN11*	*RAD50*	*RAD51*	*RAD51C*	*RAD51D*
RET	*RUNX1*	*SDHA*	*SDHAF2*	*SDHB*	*SDHC*
SDHD	*SLX4*	*SMAD4*	*SMARCA4*	*STK11*	*TERC*
TERT	*TMEM127*	*TP53*	*TSC1*	*TSC2*	*VHL*
WT1					

Table 3-3.1. The panel of the 85 cancer susceptibility genes targeted and sequenced using a custom assay developed by The University of Chicago Genetic Services Laboratory.

3.3.1.3 Somatic Mutations and Functional Tumor Studies

DNA was extracted from fresh-frozen, paraffin-embedded MM samples from two next-generation sequencing platforms, UCM-OncoPlus (N=147 gene panel) and Foundation Medicine (N=315 gene panel), in order to inspect for somatic mutations (Appendix C).

3.3.1.4 Statistical Analysis

Two-sided Fischer's exact test, Wilcoxon signed-rank test and logistic regression were used, as described above (under 3.2.1.4). Likelihood ratio tests were utilized to compare nested models and two-sided exact binomial test to compare the frequency of germline mutations. Statistical significance was reached when $p < 0.05$.

3.3.2. RESULTS

3.3.2.1 Population Demographics

There were 250 current and 12 historical patients that were taken into consideration for Study III; of those, 198 had sufficient germline DNA available and were included in the study (Figure 3-3.1). The population characteristics are summarized in Table 3-3.2. Twenty-seven of the patients (14%) had additional primary cancer diagnoses, and 173 (87%) of their first (FDR) and/or second (SDR) degree relatives, were previously diagnosed with cancer, as well (Table 3-3.3). Thirteen of the patients of this cohort had one or more FDR or SDR with MM.

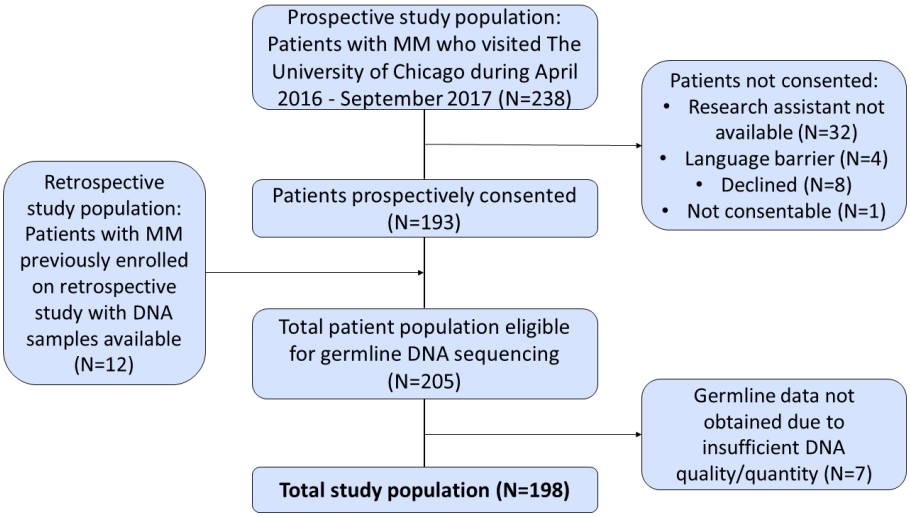


Figure 3-3.1. Consort diagram for Study III.

	N (%)
Total	198 (100)
Sex	
Male	136 (69)
Age at diagnosis, median (IQR)	67 (59, 73)
Ethnicity	
Non-Hispanic white	192 (97)
Black	3 (2)
Asian	3 (2)
Site of origin	
Pleura	148 (75)
Peritoneum	44 (22)
Pleura & Peritoneum	3 (2)
Tunica vaginalis	3 (2)
Histology	
Epithelioid	157 (79)
Sarcomatoid	13 (7)
Biphasic	23 (12)
Unknown	5 (3)
Additional cancer primary*	
Yes^	27 (14)
Hematologic	8 (4)
Breast	7 (4)
Prostate	5 (3)
Melanoma	4 (2)
Colon	2 (1)
Renal	2 (1)
Other	3 (2)
FDR with cancer*	
Yes	142 (72)
No	54 (27)
Unknown	2 (1)
FDR and/or SDR with cancer *	
Yes	173 (87)
No	23 (12)
Unknown	2 (1)
Asbestos exposure	
Definite	104 (53)

Probable	22 (11)
Possible	35 (18)
None	35 (18)
Unknown	2 (1)
Type of asbestos exposure#	
Primary	98 (49)
Secondary	32 (16)
Primary & Secondary	31 (16)
Smoking status	
Current	1 (1)
Former	89 (45)
Never	106 (54)
Unknown	2 (1)
Treatments received for MM	
Curative intent surgery	100 (51)
Chemotherapy	165 (83)
Platinum-based chemotherapy	159 (80)

*excludes non-melanoma skin cancer
^27 subjects had 31 total additional cancer primaries; other includes ovarian cancer (1), Wilm's tumor (1), and gastrointestinal stromal tumor (1)
#Asbestos exposure type for the N=161 subjects with possible, probable, or definite exposure

Table 3-3.2. Patient characteristics

Cancer Type	FDR	FDR and/or SDR
Breast	41	67
Colorectal	30	46
Lung	27	53
Prostate	27	40
Melanoma	10	11
Leukaemia	10	15
Brain	9	16
Lymphoma	8	14
Mesothelioma	8	14
Pancreas	8	13
Ovarian	8	11
Hepatic	7	9
Head & Neck	6	12
Thyroid	6	8
Urinary tract	6	8
Renal	5	8
Uterine	5	8
Multiple myeloma	4	5
Cervical	3	4
Gastric	2	12
Retinal	1	2
Bone	1	3
Wilm's tumor	1	1

Table 3-3.3. Total number of first- (FDR) and/or second-degree (SDR) relatives with specific cancers in family histories from patients with MM

3.3.2.2 Germline Mutations

The analysis identified 24 germline mutations in 23 (12%) of the 198 MM patients (one patient carried two germline mutation, one in *BAP1* and one in *TMEM127*). There were 13 different genes that presented with the 24 mutations, with *BAP1* the most common (N=6, 25%) (Figure 3-3.2). Three of the 13 families with more than one MM cases carried a germline mutation, all in *BAP1*.

Certain clinical characteristics could predict the presence of germline mutations, including peritoneal disease, limited or no asbestos exposure, second cancer diagnosis and younger age (Figure 3-3.3, Table 3-3.4, Table 3-3.5). There was no significant

association between germline mutations and sex, histology, FDR/SDR with cancer, including MM, and smoking status.

A germline mutation in *BAP1* (OR=1.658, 95%CI=[199, 76,224], p= 0.001), *BRCA2* (OR=5, 95%CI=[1.0, 14.7], p=0.03), *CDKN2A* (OR=53, 95%CI=[6, 249], p=0.001), *TMEM127* (OR=88, 95%CI=[1.7, 1,105], p=0.01), *VHL* (OR=51, 95%CI=[1.1, 453], p=0.02), and *WT1* (OR=20, 95%CI=[0.5, 135], p=0.049) was significantly more frequent in our study population compared to the non-cancer ExAC population (Table 3-3.6).

3.3.2.3 Somatic mutations

There were 54 patients with available MM tissue to undergo tumor sequencing, hereof 37 MPM and 17 MPeM samples. The most common acquired mutations were identified in *BAP1*, in 13 MPM (43%) and 11 MPeM (65%) specimens, and only two out of the 31 different mutations (6%) were germline (Figure 3-3.4 and Appendix C). Acquired mutations were also frequent in *CDKN2A* (N=10, 19% of N=54), *NF2* (N=10, 19% of N=54), *SETD2* (N= 6, 11% of N=54), *DDX3X* (N=4, 7% of N=54), and *FBXW7* (N= 4, 7% of N=54) for both MPM and MPeM, while *TP53* was only mutated in MPM cases (N=7, 19% of N=37). Interestingly, one or more germline or acquired mutations in a homologous recombination (HR) DNA repair pathway gene was found in 29/54 (52%) samples. Five of the patients with germline mutations had available tumor tissue for sequencing. Two of them had an inherited *BAP1* mutation and the other three had a *WT1*, *CHEK2* and *ATM* mutation, respectively (Appendix C, UC016, 059, 041, 102, and 170). In the tumor samples of the mutation carriers, 0-3 somatic mutations were discovered; both patients with germline *BAP1* mutations and the individual with the *WT1* mutation acquired a second pathogenic *BAP1* mutation.

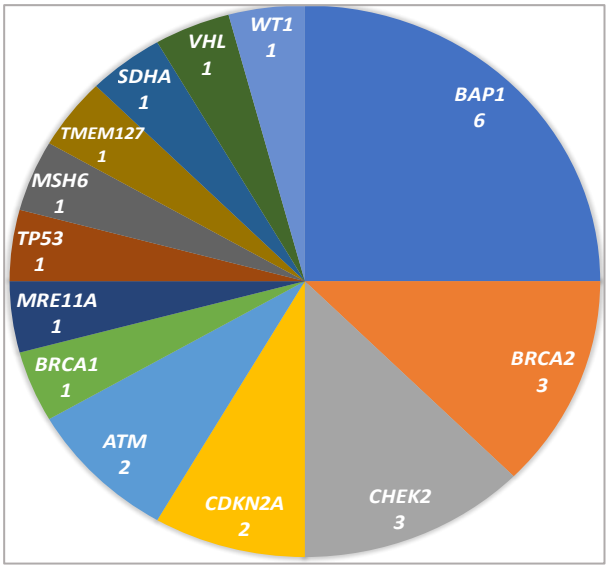


Figure 3-3.2. Twenty-four germline mutations were identified in 13 different genes in 23 patients (12% of N=198).

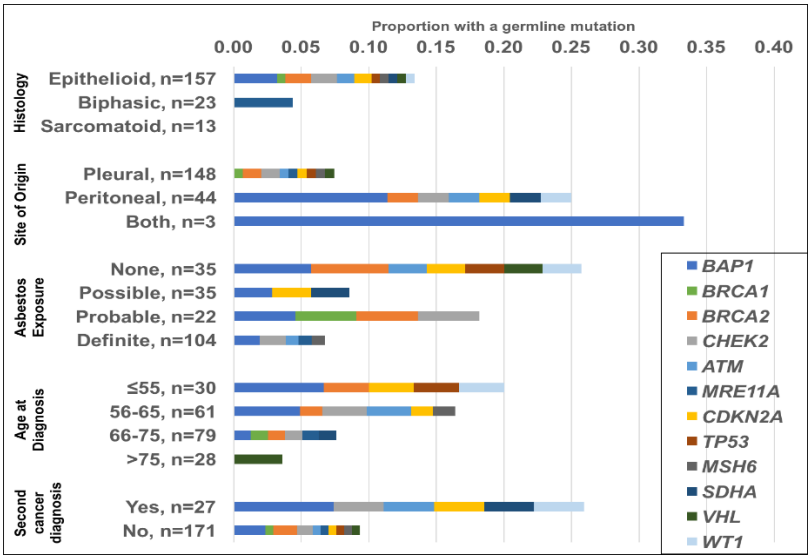


Figure 3-3.3. Proportions of the germline mutation-carriers per clinical features.

	Germline	No germline	P-value
Total, No. (%)	**23 (12)**	**175 (88)**	
Sex			
Female	9 (39)	53 (30)	0.47
Male	14 (61)	122 (70)	
Age at diagnosis, median (IQR)	61 (56, 71)	67 (59, 73)	**0.04**
Site of origin			
Pleura	11 (48)	137 (78)	**0.01**
Peritoneum	11 (48)	33 (18)	
Pleura & Peritoneum	1 (4)	2 (1)	
Tunica vaginalis	0 (0)	3 (2)	
Histology (N=193)			
Epithelioid	21 (95)	136 (80)	0.26
Sarcomatoid	0 (0)	13 (7)	
Biphasic	1 (5)	22 (13)	
Additional cancer primary**			
Yes	7 (30)	20 (11)	**0.02**
No	16 (70)	155 (89)	
FDR with cancer**			
Yes	17 (74)	119 (69)	0.81
No	6 (26)	54 (31)	
Asbestos exposure (N=196)*			
Definite	7 (30)	97 (56)	**0.02**
Probable	4 (17)	18 (10)	
Possible	3 (13)	32 (19)	
None	9 (39)	26 (15)	
Type of asbestos exposure			
Primary	7 (50)	91 (62)	0.62
Secondary	4 (29)	28 (19)	
Primary & Secondary	3 (21)	28 (19)	
Smoking status (N=195)*			
Current	0 (0)	1 (1)	0.28
Former	7 (30)	82 (47)	
Never	16 (70)	90 (52)	

**Patients with missing values excluded.. **Excludes non-melanoma skin cancer.*

Table 3-3.4. Clinical characteristics of germline mutation carriers and non-mutation carriers.

Variable	OR (95% CI)	P-value	M.1 OR (95% CI)	P-value	M.2 OR (95% CI)	P-value	M.3 OR (95% CI)	P-value
Male	0.69 (0.28-1.70)	0.42						
Histology~								
Epithelioid	1.00							
Sarcomatoid	1.00							
Biphasic	0.29 (0.04-2.25)	0.24						
Age, median*	0.95 (0.92-0.99)	**0.01**	0.96 (0.92-0.99)	**0.02**			0.97 (0.93-1.01)	0.13
Site of origin^								
Peritoneum or Both	1.00				1.00		1.00	
Pleura	0.23 (0.10-0.58)	**0.002**			0.31 (0.12-0.80)	**0.02**	0.44 (0.15-1.27)	0.13
Second cancer	3.33 (1.22-9.07)	**0.019**	3.45 (1.18-10.15)	**0.02**	2.27 (0.77-6.70)	0.14	2.73 (0.89-8.39)	0.08
Asbestos exposure	0.28 (0.11-0.72)	**0.008**	0.33 (0.12-0.89)	**0.03**	0.34 (0.13-0.90)	**0.03**	0.35 (0.13-0.95)	**0.04**

M.1: Age, Asbestos & Second cancer

M. 2: Site of origin, Asbestos & Second cancer

M.3: Age, Site of origin, Asbestos & Second cancer

~Sarcomatoid category has zero germline mutation carriers

*Age centered around median of 67 years

^tunica vaginalis cases N=3 were excluded from these analyses

#Nested models (M.1 vs M.3 and M.2 vs M.3) were compared using likelihood ratio tests; Addition of the site of origin in either comparison did not improve model fit (p=0.13)

Table 3-3.5. Predictors of a germline mutation among patients with MM.

	University of Chicago Patients with MM N=198		ExAC Non-Cancer Population N=53,105*		University of Chicago versus ExAC population	
Gene	***Mutated alleles (N)***	***Proportion of individuals with a mutation***	***Mutated alleles (N)***	***Proportion of individuals with a mutation***	***OR (95% CI)***	***P-value *****
BAP1	6	0.0303	1	0.0303	1657.5 (199-76,224)	**<.001^**
BRCA2	3	0.0152	167	0.0152	4.9 (1.0-14.7)	**0.03**
CDKN2A	2	0.0101	10	0.0101	52.6 (6-249)	**<.001^**
TMEM127	1	0.0051	3	0.0051	88.4 (1.7-1,105)	**0.01**
VHL	1	0.0051	5	0.0051	50.5 (1.1-453)	**0.02**
WT1	1	0.0051	13	0.0051	20.1 (0.5-135)	**0.049**
ATM	2	0.0101	155	0.002929	3.5 (0.4-12.9)	0.12
CHEK2	3	0.0152	770	0.015480	0.98 (0.2-2.9)	1.00
BRCA1	1	0.0051	102	0.002000	2.5 (0.1-14.6)	0.33
MRE11A	1	0.0051	33	0.000625	8.1 (0.2-49)	0.12
TP53	1	0.0051	29	0.000548	9.3 (0.2-56.4)	0.10
MSH6	1	0.0051	102	0.001934	2.6 (0.1-15)	0.32
SDHA	1	0.0051	53	0.001026	4.9 (0.1-29)	0.18

**Number of individuals sequenced varies by genomic position*

***Two-sided exact binomial tests without adjustment for multiple testing*

^Remain significant at alpha<0.004 if Bonferroni correction is used

Table 3-3.6. Mutation frequencies in patients with malignant mesothelioma versus a non-cancer population estimate

Unique Patient Identifier
Origin*
Histology**
BAP1
BAP1 VUS
CDKN2A
NF2
TP53
SETD2
DDX3X
FBXW7
PTEN
PBRM1
NOTCH1
FANCA
PIK3CA
KDM6A
CSF1R
TERT
EPHA5
WT1
ATM
ATR
PTPN11
BRCA2
DNMT3A
RB1
CTNNB1
CARD11
AKT2
CCND3
FOXP1
MITF
EPHB1
ASXL1
NF1
NRAS
MLH3
MDM4
SMARCA4
KDR
MSH6
CHEK2
Total Pathogenic Acquired Mutations
Proportions
Pleura (% of n=37)
Peritoneal (% of n=17)
Median number of mutations per tumor

**Origin: Dark blue=pleural; light blue=peritoneal*
***Histology: Dark red=epithelioid, pink=biphasic, green=sarcomatoid*
Abbreviations: VUS=variant of uncertain significance
Mutation types: loss, large deletion or duplication, nonsense, frameshift, splice site=dark gray; missense, in frame deletion, promoter mutation=green; amplification= blue; germline variants are notated by a ★.
Tumors with multiple variants in the same gene are notated with the number of unique variants identified.

Figure 3-3.4. Genetic variants identified by site of origin and histology in 54 MM specimens

3.3.3. CONCLUSION

Study III identified 13 different genes that presented with 24 germline mutations in 23/198 MM patients. The spectrum included inherited mutations in the well-characterized gene *BAP1*, in previously reported genes in case reports (*BRCA2*, *CDKN2A*, *ATM*, *BRCA1*, *TP53*, *MSH6*) and novel genes in the MM context (*TMEM127*, *CHEK2*, *MRE11A*, *VHL*, *WT1*, and *SDHA*) (Aim 5). Germline mutations in *BAP1*, *BRCA2*, *CDKN2A*, *TMEM127* and *WT1* were overrepresented among the MM patients in comparison to a non-cancer population (Aim 5). The study concluded that peritoneal disease, no known asbestos exposure, second cancer diagnosis and younger age were significant predictors of a germline mutation (Aim 6). The HR DNA repair pathway was implicated in more than half of the MM cases, either as a result of a germline or a somatic mutation (Aim 7).

CHAPTER 4. DISCUSSION

The aims and the main conclusions, as well as the clinical impact and the methodological considerations of the thesis will be discussed in this chapter.

4.1. AIM 1

To summarize the most important current and most promising future biomarkers within the diagnosis, prognosis and prediction for MM.

Study I attempted to assess Aim 1 by providing an overview of the most important studies and describing the biomarkers that are currently essential to the MPM diagnosis, as well as the most promising diagnostic, prognostic and predictive biomarkers. The evident diverging results of the numerous studies about MM biomarkers are a consequence of the lack of standardized treatments and assays, the rarity and aggressiveness of the disease and the limited number of patients that hamper the conclusiveness of the results. The consensus statement for the pathologic MM diagnosis established by the International Mesothelioma Interest Group included typically calretinin and one other positive marker (e.g. cytokeratin 5, podoplanin or WT1) and two negative markers (149). The consensus statement and the four-biomarker-panel have enhanced the accuracy of the immunohistochemical classification but the available positive markers are of limited sensitivity. Calretinin is one of the most valuable immunohistochemical markers for the MM diagnosis and it was routinely introduced in the pathologic laboratories in the late 1990s (149). However, calretinin is negative in half of the cases of sarcomatoid MM, while it can also be positive in some cases of lung adenocarcinoma, breast, serous, renal cell, small cell and squamous cell carcinoma (focal reaction) (150,151). Similarly, cytokeratin 5 is positive in all squamous cell, and occasionally in other carcinomas, while benign fibrosing lesion may express podoplanin (89,90,92,93). WT1 has no value in the differential diagnosis of breast cancer and serous carcinomas of the ovary and peritoneum (95).

Some potential biomarkers or panels of biomarkers for the diagnosis, prognosis, prediction and early detection of MM have shown interesting results and warrant further investigation. Furthermore, several biomarkers have been proposed as a cost-effective means of treatment but there are contradicting findings as to their sensitivity and specificity (126,152). Mesothelin, the most promising current MM biomarker and the only one that is commercialized, is specific but not sensitive, as it can be elevated in other malignant and non-malignant diseases, such as renal failure, as well (116,122). Moreover, it is documented that mesothelin serum levels can decrease at response to treatment with tumor shrinkage in some (but far from all) patients;

however, it is not of clinical utility due to the low robustness (116). The role of mesothelin in MM screening has also been investigated with overall discouraging results (116). The diagnostic utility of osteopontin is controversial, as some studies have shown that osteopontin levels have prognostic and/or predictive value and high levels of osteopontin were individually associated with worse prognosis, but other researchers did not reach the same conclusions (124,125). Fibulin-3 has shown potential in distinguishing MPM patients from an asbestos-exposed non-cancer population and in monitoring the progress of the disease, but the same findings were not confirmed in subsequent studies (133,153). Hyaluronate is prevalent in progressed MPM but it is also elevated in other pathologies, such as inflammatory joint disease and hepatic fibrosis, thus its diagnostic utility is limited (154). Several micro-RNAs have been suggested by profiling studies but only two microRNA signatures (miR-16-5p/miR-126-3p/miR-143-3p/miR-145-5p/miR-192-5p/miR-193a-3p/miR-200b-3p/miR-203a-3p/miR-652-3p and miR-126-3p/miR-103a-3p/miR-625-3p/mesothelin) proved to be of value as to early diagnosis according to a meta-analysis and functional studies, while large-scale validation is required (155). The role of long non coding RNA in MPM is being investigated the recent years but is yet to be fully understood (156). The lack of validation for proteomics, gene expression profiling, deformability cytometry, fluorescence in situ hybridization assay and selected reaction monitoring assay technology impedes their use in the clinic. In conclusion, non-invasive markers of higher sensitivity and specificity are being explored but most of them are not further assessed beyond the initial discovery phase (126,152). Therefore, their potential is limited and their clinical use is unlikely in the near future. However, prognostic and predictive biomarkers able to separate responders from non-responders would assist clinicians in the management of MM, and they would spare the patients from ineffective treatment and the society from unnecessary costs (126,152). Furthermore, biomarkers that would enable the detection of the high-risk asbestos exposed individuals, would facilitate the early diagnosis of the disease.

In summary, the current diagnostic, prognostic and predictive MM biomarkers are inadequate but new are constantly emerging. Hence, there is a need for further research in order to discover new, and large prospective studies in order to validate the prevailing biomarkers for MM.

4.2. AIM 2

To explore the extent and impact of non-occupational asbestos exposure for women and men with MM.

Aim 2 was investigated in Study IIa and IIb. We concluded that 25% of the total population had non-occupational exposure to asbestos, including 60 patients (14%) with sole environmental exposure. In particular, 23% of the women and 12% of the

men had a sole environmental exposure, whereas 57% of the male patients had a combined occupational and environmental exposure. A ´´hotspot´´ of 20 parishes with shared borders within 10km from asbestos plants in the city of Aalborg was defined in Study IIa, where the incidence and RR of MM for women was higher than the rest of Denmark. Particularly, the parish, where DAF was located presented with the highest incidence and RR for MM. A corresponding ´´hotspot´´ of 16 continuous parishes within a 10km radius from asbestos industries was identified through Study IIb, where the cumulative incidence of MM for men was increased compared to the rest of the Region of North Denmark. Ten parishes, whose male residents had higher RR of MM than the Danish men, were also part of this ´´hotspot´´. The parish where Aalborg shipyard was located had the highest RR of all and the parish where DAF operated, presented with the fourth highest RR.

Environmental exposure to asbestos is often neglected and under-reported and MM patients with this type of exposure are not entitled to financial compensation. The most comprehensive studies regarding environmental asbestos exposure and MM risk come from the Italian national registry of MM, and especially three cities in northwest Italy, Casale Monferrato, Bari and Broni (21,28,157,158). These cities have had heavy asbestos burden, as large asbestos cement plants were operating for decades in densely populated areas and thus, the residents have suffered a high MM incidence without direct occupational exposure. However, the impact of heavy asbestos industry operating in the city of Aalborg for more than six decades had not been evaluated before. The afore-mentioned studies describe MM ´´hotspots´´ around asbestos industries and usual characteristics of environmental exposure, such as long disease latency and duration of exposure, which are observed in our cohort, as well. These findings corroborate that large-scale environmental asbestos contamination took place in Aalborg. This extended environmental asbestos pollution contributed to the increased incidence and RR of MM for both men and women around asbestos industries in Aalborg in comparison to less contaminated geographical areas.

The role of domestic asbestos exposure in MM carcinogenesis has been documented in the literature but the detailed study of MM due to this type of exposure is obstructed due to the long latency period and the small number of patients (20,159,160). Several hundred MM cases have been reported among family members of asbestos workers and studies have shown that there is a five-fold increased risk of MM for case-control studies and 8.5-fold risk for cohort studies of domestically exposed individuals (23,161). The family members include typically fathers, brothers and sons that work as miners, manufacturers of asbestos-containing products, insulators, shipyard and construction workers, which is concordant with our findings (23,24). The fiber type is not always taken into account in the published research, but the majority of the studies describe MM patients that have been domestically exposed to amphiboles; thus the impact of household exposure to chrysotile is under-investigated (24). This is not the case with our cohort, where one fourth of the women had been domestically exposed to chrysotile asbestos, as their relatives were employed at DAF and

construction industry. The long latency of 20-70 years is responsible for the constantly increasing MM incidence for the population of the North Denmark Region. As asbestos was banned in the late 1980´s, the incidence peak is yet to be reached.

In summary, our observations strongly suggest that non-occupational asbestos exposure has had a major impact on the male and female population of the Region of North Denmark, with the population at the highest risk residing within 10 km from asbestos industries. According to our findings, the clinicians need to be cautious with the assessment of the risk of MM as a result of non-occupational exposure to asbestos. To our knowledge, this is the first large study to thoroughly evaluate the effect of asbestos exposure for the total population of the Region of North Denmark and the first of its kind in the Scandinavian countries. Study IIa along with previous research of our research group members led to the re-evaluation of the rules of financial compensation for MM patients that have been domestically exposed to asbestos in Denmark.

4.3. AIM 3

To elucidate and compare the asbestos exposure patterns for the male and the female MM patients.

The asbestos exposure profiles of the women and men with MM were examined in Study IIa and IIb, respectively, while the comparison of the two profiles was performed in Study IIb. The vast majority of the women had a sole or combined non-occupational exposure to asbestos, while only 9% of them had worked with asbestos. On the contrary, 82% of their male counterparts had a sole or combined occupational asbestos exposure (male:female ratio of 34:1 for occupational exposure). On the other hand, sole or combined domestic exposure to asbestos was common for the female but very uncommon for the male population (44% versus 1%, respectively and a male:female ratio of 1:8). In the literature, several cohorts are encountered, where the women have a high rate of non-occupational and the men of occupational asbestos exposure (40,162,163). However, the small fraction of occupationally exposed women of this population is atypical. This is probably a direct aftermath of the fact that, historically, the asbestos workers in the Region of North Denmark have been men. The jobs of the occupationally exposed patients are similar to the previously reported high-risk occupations, such as workers at a shipyard, the construction industry and the DAF (40,164,165). The finding that 25% of the MM patients were employed at DAF and construction industry presents with a particular interest, as it confirms that chrysotile is definitely carcinogenetic, in line with the World Health Organization and in contrast to some researchers that even recently question the role of chrysotile in MM tumorigenesis (166–168). Environmental asbestos exposure is present at both groups with a male:female ratio of 1.9:1. A male:female ratio close to

1 would be expected in this cohort, as both men and women were equally subjected to the environmental asbestos contamination. Unregistered recreational use of asbestos could account for some of the environmental MM cases among men. There is also a small difference regarding the unknown exposure, as it is more frequent among women compared to men. A high rate of unknown exposure for female MM patients has been described in previous studies, but one needs to address this observation cautiously, as it is not clear whether there is a real absence of asbestos exposure or if the non-occupational asbestos exposure sources were poorly evaluated (40,54,169,170). Interestingly, more than half of the men and more than one third of the women had a combined asbestos exposure. The most frequent combined exposures were occupational and environmental for men, and domestic and environmental for women. The fact that more than 50% of the male and female MM patients were born and raised within 10km from DAF and Aalborg shipyard is the reason for this observation. The potential contribution of the extra burden of combined environmental exposure to the high cumulative incidence and RR of MM inside the ´´hotspot´´ is difficult to assess. There are not many studies identifying combined exposure to asbestos and as result, the consequences hereof are not known.

In summary, the men and women of the Region of North Denmark had profoundly different exposure profiles, with domestic and/or environmental exposure being most common for the female and occupational and/or environmental exposure for the male cohort. Combined exposure was very frequent for both populations.

4.4. AIM 4

To examine whether the histopathological MM subtype, epithelioid or non-epitheliod, and the MM location, pleura or peritoneum, are associated with the type of asbestos exposure or the gender of the patient.

Both Study IIa and Study IIb attempted to elucidate this matter. The fact that the type of asbestos exposure could affect the development of MPeM or MPM in women (Study IIa), indicates that more intense, occupational exposure may predispose to peritoneal while lighter, non-occupational exposure to pleural disease. There are previous studies that reached the same conclusion, but the retrospective nature of the study and the relatively small number of patients call for further validation (171,172). Study IIb showed that MPeM was overrepresented among women in comparison to men. This could be associated with the different pathways that are involved in MPeM carcinogenesis and might imply a general susceptibility of women to MPeM due to hormonal, anatomical or genetic dissimilarities (40,173). Additionally, the epithelioid MM subtype was linked to non-occupational, while the non-epithelioid subtype to occupational asbestos exposure, which suggests that less intense (non-occupational) asbestos exposure might predispose for the less aggressive epithelioid MM subtype

and vice versa (101). The literature presents contradicting results on the subject. Some studies have illustrated that heavier and longer asbestos exposure could predispose to non-epithelioid subtypes, but others found no association between the frequency or intensity of asbestos exposure and the histopathological MM subtype (5–7).

In summary, there are indications that the type of asbestos exposure might influence the MM location in women. Similarly, the development of pleural or peritoneal disease seems to be influenced by gender in the total population, whereas the development of epithelioid or non-epithelioid subtype might be affected by the type of asbestos exposure. However, validation of these hypotheses on larger populations is necessary before definitive conclusions can be drawn.

4.5. AIM 5

To assess the prevalence and the spectrum of germline mutations in MM.

Aim 5 was addressed in Study III. MM was considered to be a highly exposure based tumor but we found that 12% of the population carried germline mutations in cancer susceptibility genes. The prevalence described for other malignancies, e.g. metastatic prostate cancer, is quite comparable to the identified proportion of germline mutations in MM, substantiating our observation (174). The well-described *BAP1* was responsible for 25% of the mutations, but 12 additional genes were identified, as well, whilst six of them were not previously associated with MM. This finding could provide an explanation for MM patients that presented with a suspicious personal or family history of malignancy but had no inherited *BAP1* mutation; a germline mutation in another gene could be the case. Half of the identified genes (*BAP1*, *BRCA2*, *CDKN2A*, *TMEM127*, *VHL* and *WT1*) were significantly overrepresented in the MM versus a non-cancer population, supporting the hypothesis that inherited predisposition has a causative role in MM tumorigenesis. This is demonstrated for other malignancies, e.g. breast and ovarian cancer, where pathogenic genes were found to be overrepresented in cancer patients in comparison to the non-cancer population, but it was never before reported for MM (174,175). In general, the literature describing inherited mutations for MM patients in genes other than *BAP1* is very limited. Inherited mutations in *ATM*, *CDKN2A*, *BRCA1*, *BRCA2*, *MSH6*, *MLH1*, *PALB2*, and *TP53* have been reported in individual patients but their prevalence was not assessed (70,71,176). Recently, Betti et al. detected germline mutations in DNA repair genes in 9.7% of a MM population, including *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *FANCI*, *FANCC*, *FANCF* and *SLX4* (69). The authors hypothesized that DNA damage could not be repaired because of the genes defect, which resulted in MM genesis (69). However, no previous study has provided sufficient proof of causation between MM and inherited mutations besides *BAP1*. Emerging research is contributing to the identification of the full spectrum of inherited predisposition for MM.

In summary, the spectrum of germline mutations in MM includes 13 cancer susceptibility genes, where *BAP1* accounts for one fourth of the cases. The prevalence of these mutations is higher than previously reported, indicating that germline mutations might be a universal feature of substantial subset of all cancers, including MM. This is the first large-scale sequencing study of germline mutations in MM demonstrating the causative role of susceptibility genes for MM (besides *BAP1*) and its findings were supported by subsequent research.

4.6. AIM 6

To determine disease characteristics that can predict the presence of a germline mutation.

Study III shed light on this question. Clinical features that could predict the presence of a germline mutation include limited or no asbestos exposure, peritoneal disease, a previous second cancer diagnosis and younger age. Limited or no asbestos exposure has been established for *BAP1* mutation carriers and is confirmed for all the mutation carriers by our data (177). A pathogenic gene alteration could be *de novo* carcinogenetic even in the absence of asbestos exposure or it might be a result of gene-environment interaction. This gene-environment interaction has been previously described in MPeM, as well, in the context of its weaker association with asbestos exposure in comparison to MPM (178–180). In our findings, it is noteworthy that 25% of the MPeM patients presented with a genomic alteration versus 7% of the MPM patients. This increased prevalence indicates that genetic susceptibility may play a more important part in MPeM pathogenesis than in MPM. The fact that mutation carriers develop MM at a younger age and have significantly more additional primary malignancies is not surprising; inherited mutations are known to be linked with higher risk of cancer and early-onset cancer diagnosis (181). Clinical features that can predict an inherited mutation are infrequently reported; early age at MM onset and no asbestos exposure are the two common characteristics that have been described for *BAP1* mutation carriers (182,183). There are also studies that imply an increased susceptibility for MM for individuals with FDRs with MM and second primary cancer diagnosis; however, these studies did not assess the genetic status of the patients (184,185).

In summary, we conclude that MM patients who present with limited asbestos exposure, peritoneal disease, history of additional primary malignancies and early-onset MM diagnosis should be suspected for inherited MM susceptibility and they should be offered genetic counselling. The current genetic guidelines need to be reevaluated, though, as only half of the mutation carriers of this cohort would be identified by the use of the standard genetic tests. Comprehensive genetic testing

would allow us to identify the individuals that are in high-risk of developing a malignancy and, thus, to achieve early detection.

4.7. AIM 7

To explore genetic pathways in MM carcinogenesis.

Potential genetic pathways in MM carcinogenesis were determined in Study III. More than half of study´s population had inherited or acquired defects in genes involved in the HR-mediated DNA repair pathway. Six genes with a role in this pathway carried germline alterations, *BAP1, BRCA1, BRCA2, CHEK2, ATM* and *MRE11A.* Somatic mutations were found in HR genes, as well, among others in *BAP1, SETD2, FANCA* and *TP53*. *BAP1* mutations inhibit double strand breaks and affect the accumulation of proteins involved in the HR-mediated DNA damage process, while *BAP1* loss is associated with increased sensitivity to Poly(ADP-ribose) polymerase inhibitors (PARPi) (186). *BRCA1*, *BRCA2*, *FANCA* and *TP53* contribute to homology-directed repair of double strand breaks (187–189). *CHEK2* encodes a protein that, when activated is known to stabilize the tumor suppressor protein p53, leading to cell cycle arrest (190). In addition, this protein interacts with and phosphorylates *BRCA1*, allowing *BRCA1* to restore survival after DNA damage (191). *ATM* encodes an effector kinase, which regulates the activities of downstream checkpoint proteins in the HR pathway and *MRE11A* encodes a nuclear protein involved in HR, telomere length maintenance, and double strand breaks repair (192,193). *SETD2* is required for the repair of double strand breaks and the activation of *ATM* (194). *WT1* might be related, as well, by promoting HR-mediated DNA damage repair (195). Inherited and acquired mutations in genes involved in the mismatch repair pathway were also found (*MSH6* and *MLH3*). Of the remaining genes with inherited mutations, *VHL* and *SDHA* induce carcinogenesis by modifying the hypoxia-inducible factor expression, while *TMEM127* controls cell proliferation acting as a negative regulator of Target of Rapamycin signaling pathway; these constitute MM pathways that warrant investigation (196–198).

The observation that a high rate of the mutated genes encode proteins involved in the HR-mediated DNA repair pathway has some interesting clinical implications as to potential chemotherapeutic targets. Alterations of the DNA repair system, either in germline or somatic cells can affect the prognosis and the effect of DNA damaging agents. Patients with breast, ovarian and prostate cancer that are carriers of *BRCA1* and *BRCA2* mutations have a better prognosis and response when treated with cisplatin (175,199–201). PARPi are used for these patient groups, as they are highly selective for tumor tissues, which are completely BRCA deficient, compared with normal tissues that are heterozygous at the BRCA locus (202,203). Tumors with both germline and somatic *BAP1* alterations, resulting in total loss of the *BAP1* function

have also been described to be sensitive to PARPi (204). Therefore, it is possible that MM patients with germline mutations in a DNA repair gene could also benefit from treatment protocols that include PARPi. Currently, two ongoing clinical trials of the PARPi olaparib and naraparib (Clinicaltrials.gov NCT03531840 and Clinicltrials.gov NCT03207347) are including patients with MM in an attempt to assess the efficacy of these agents in MM treatment. Additional clinical trials aiming to evaluate the effect of PARPi in MM patients with germline mutations would also be relevant. Furthermore, patients carrying germline mutations in *BAP1* and other genes have often prolonged survival in comparison to non-mutation carriers (183). Thus, a germline mutation in a DNA repair gene could also serve as a prognostic marker for MM management.

In summary, we describe novel germline mutations that could be implicated in the MM tumorigenesis, where the most common are genes encoding proteins of the HR-mediated DNA repair pathway. Our findings may justify the sensitivity of cisplatin in MM and support the idea that PARPi could be an effective therapeutic strategy in MM (69,205).

4.8. METHODOLOGICAL CONSIDERATIONS

4.8.1. DOCUMENTATION OF ASBESTOS EXPOSURE

One of this thesis' primary methodological considerations is linked to the documentation of asbestos exposure. The Danish registries are a robust source of information due to the high-quality and validated data they enclose. However, the Danish Supplementary Pension Fund Registry includes occupational information after 1964, thus the detailed professional history of the patients and their relatives before 1964 was not available. The most active period of the asbestos industry was, though, after 1945, especially after 1960 (Figure 1-9.2), and the research group supplemented the occupational history by incorporating information about the patients´ work history through their medical records; hence, the loss of information is limited. Nonetheless, undocumented asbestos exposure could have taken place for some individuals of this cohort in relation to recreational use of asbestos or from damaged asbestos-containing buildings. It was not possible to exclude such exposure for the total population, as asbestos was a popular material in Denmark and it was broadly used until the late 1980´s. As a result, misclassification of asbestos exposure for some individuals could have occurred. However, recreational asbestos use was probably distributed equally and this potential misclassification would be low-scale and it would not affect the main conclusions of the thesis.

4.8.2. ISOLATED PARISHES OUTSIDE THE ´´HOTSPOT´´

A high cumulative incidence and relative risk of MM was observed in isolated parishes in the Region of North Denmark, outside the 10km radius from asbestos industry. All these parishes form no hotspot and have one or two MM cases that developed in a small population (N<900). Hence, no statistically significant conclusions can be drawn from this observation. Retrospectively, we acknowledge that the use of Bayesian smoothing should have been considered, as it could possibly have been able to smear out these singular cases. However, a ´´hotspot´´ around the city of Aalborg would still be evident and thereby, the main conclusion would not have been altered.

4.8.3. RARE CAUSES OF MALIGNANT MESOTHELIOMA

It was not possible to investigate the population of Study II for rare causes of MM, such as exposure to erionite, radiation and genetic susceptibility. Erionite is not encountered in the Region of North Denmark and therefore, we are confident that this is not a source of bias for the present cohort. Radiation and heredity are both rare causes, which have not been reported in the Region of North Denmark to our knowledge, but we cannot rule out that there could be single cases. In total, the information sources used in Study II are of high-quality, without recall bias, but from compulsory and validated registries, and the MM diagnosis for all the patients was confirmed. Hence, the main findings and conclusions are substantiated.

4.8.4. INTERPRETATION OF THE GENETIC TESTING

A conservative approach of the genetic testing in Study III was chosen and only pathogenic or likely pathogenic inherited mutations were investigated. Similarly, the panels used to detect acquired mutations did not include all the genes that are involved in the HR DNA pathway. Furthermore, both the platforms that were utilized may be unable to spot small copy number changes. As a result, there could be more patients carrying a pathogenic germline or somatic mutation that were not reported.

4.8.5. SELECTION BIAS

The men and women that were included in Study III attended a tertiary referral center, which encloses referral bias. The population of tertiary-care centers consists mostly of younger patients with a good performance status, as elderly and feeble individuals

are unlikely to be referred. Besides, the asbestos exposure and the familial history of cancer in Study II was self-reported, thus recall bias is possible. However, the finding that limited asbestos exposure and family history of malignancy are associated with higher odds of carrying a germline mutation is concordant with previous and subsequent studies.

4.8.6. DIRECT EVIDENCE OF CAUSATION

There was available tissue for sequencing from only five of the patients with germline mutations. This limitation did not allow us to perform further functional tests on tumor specimens in order to support the causation of MM genesis. In addition, all the patients, whose families presented with more than one MM cases, carried a germline *BAP1* mutation. Our hypothesis that germline mutations in *BRCA2*, *CDKN2A*, *TMEM127*, *VHL* and W*T1* have a causal relation with MM pathogenesis would be further supported if one or more of those genes were found to be mutated in familial MM cases.

4.9. CONCLUSION

This thesis reaches notable conclusions regarding risk factors for MM. Historically, MM was mainly attributed to occupational exposure to asbestos, but Study II demonstrates that non-occupational exposure seems to be the primary cause of MM for women and may be implicated in MM tumorigenesis for the majority of the men. A ´´hotspot´´ within 10km of asbestos plants stands out, where the highest cumulative incidence and relative risk of MM for women and men is noticed. Interestingly, the male and female MM patients have significantly different exposure patterns, but combined exposures (e.g. occupational and environmental or domestic and environmental), which are rarely documented, are common for both populations. Study III highlights that germline mutations are probably involved in MM susceptibility at a higher degree than was previously believed. Inherited mutations in 13 cancer susceptibility genes were identified for 12% of a MM population. Six of these genes are overrepresented among MM patients in comparison to a non-cancer population, which supports their causative role in MM carcinogenesis. Furthermore, Study III describes limited asbestos exposure, peritoneal disease, history of additional primary malignancies and early-onset MM diagnosis as clinical predictors of a germline mutation. In addition, more than half of the mutation carriers have an inherited or acquired genetic defect in the HR-mediated DNA repair pathway, which may have clinical implications as to potential chemotherapeutic targets. Lastly, this thesis outlines the established and emerging MPM biomarkers and underlines the need for validated and robust diagnostic, prognostic and predictive biomarkers to assist the

clinicians. Nonetheless, these studies have also raised some questions and have set the foundation for future research.

CHAPTER 5. FUTURE PERSPECTIVES

This thesis has generated new hypotheses to investigate and has provided the framework for future studies. The following studies are being planned:

Study 1. Patient characteristics associated with better prognosis, and identification of treatment types that increase survival.

The search of the Danish registries and the medical journals for the population of Study II provided us with useful asbestos exposure, pathological and clinical information for all the MM patients. The asbestos exposure and pathological characteristics are described above under Study II. The clinical information include survival, type of treatment, age, gender, performance status, comorbidities and stage of MM. A large retrospective study has been conducted to investigate potential prognostic factors for men and women with MM. The main focus of the study is to elucidate which patient characteristics are associated with better prognosis, and which treatment types increase survival. A manuscript is in progress and expected to be submitted in summer 2019.

Study 2. Potential prognostic and predictive MM biomarkers for chemotherapy.

We have prospectively consented 30 patients with MPM and we have gathered blood and tumor tissue samples before treatment for all of them. Some of the patients received chemotherapy, others received chemotherapy and underwent surgery, while a minority received no treatment. We have also acquired blood samples after therapy, at the time of disease progression for most of the inoperable patients, and both blood and tumor specimens at the time of surgery for the operable patients. The inclusion of patients for Study 2 is completed, but blood/tumor samples after therapy have not been gathered from all the patients yet, as some of them are currently under treatment. We are planning to test prognostic and predictive biomarkers on the patients´ tumor and blood samples and to correlate our findings with the patients´ clinical data and course of disease. The promising biomarkers that will be discovered will be validated on the retrospective material from Study II.

Study 3. Validation of potential prognostic and predictive MM biomarkers for chemotherapy.

After the reclassification of the MM samples of the population of Study II, there was available tumor tissue from 180 patients. At the same time, there are clinical and pathological data about all these patients at our disposal. Thus, we designed an exploratory study to investigate promising predictive and prognostic biomarkers for MM. The choice of the biomarkers is based on Study I and emerging research. The results of the testing of the biomarkers will be correlated with the patients´ clinical data. In order to test several biomarkers simultaneously with the minimal tissue waste, we will use tissue microarray (TMA), which has been constructed at the Institute of Pathology. We expect that this study will generate several scientific papers.

Study 4. Potential prognostic and predictive MM biomarkers for both immunotherapy and chemotherapy.

A prospective study is ongoing at The University of Chicago Medicine MM clinic, aiming to examine prognostic and predictive MM biomarkers for immunotherapy. The study is currently including patients. Both tumor tissue samples before immunotherapy and detailed clinical information for all the patients will be available. We are planning to use the same panel of prognostic and predictive biomarkers in this study and the above-described Study 2 and combine the findings of the two groups in order to investigate potential prognostic and predictive MM biomarkers for both immunotherapy and chemotherapy.

Study 5. The landscape of genetic susceptibility of asbestos induced MM in Denmark and abroad.

Blood samples of Danish MM patients as well as blood specimens from biobanks abroad will be sequenced to identify the spectrum of cancer susceptibility genes. This is a collaborative multicenter effort, including Aalborg University Hospital, Århus University Hospital and The University of Chicago MM clinic, which is currently being organized.

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Appendix A. BACKGROUND

Table 1. Represents the cases, crude rate and age/standardized rate for Malignant Pleural Mesothelioma (MPM) in Denmark (DK) and in the Region of North Denmark (ND) during 1972-2015 (data from NORDCAN).

Years	MPM cases in DK	Crude rate for DK	Age-standardized rate in DK	MPM cases in ND	Crude rate in ND	Age-standardized rate in ND
1972	42	1.7	2.2	4	1.4	1.6
1973	45	1.8	2.4	4	1.4	1.6
1974	36	1.4	1.8	3	1.1	1.0
1975	46	1.8	2.3	5	1.8	2.1
1976	26	1.0	1.2	4	1.4	1.4
1977	42	1.7	2.1	4	1.4	1.6
1978	40	1.6	2.0	3	1.1	1.2
1979	43	1.7	2.2	2	0.7	1.2
1980	55	2.2	2.8	10	3.5	4.1
1981	52	2.1	2.6	6	2.1	2.4
1982	41	1.6	1.9	6	2.1	2.2
1983	61	2.4	3.0	7	2.5	2.7
1984	54	2.1	2.5	2	0.7	0.9
1985	48	1.9	2.3	7	2.5	2.6
1986	64	2.5	3.1	9	3.2	3.2
1987	43	1.7	2.0	7	2.5	2.5
1988	60	2.4	2.8	13	4.6	5.2
1989	63	2.5	3.0	10	3.5	3.9
1990	48	1.9	2.3	6	2.1	2.5
1991	46	1.8	2.1	10	3.5	3.9
1992	67	2.6	3.2	9	3.1	3.6
1993	65	2.5	3.1	10	3.5	3.8
1994	58	2.3	2.7	7	2.4	3.0
1995	69	2.7	3.1	7	2.4	2.5
1996	63	2.4	2.8	15	5.2	5.6
1997	88	3.4	3.8	17	5.9	6.1
1998	67	2.6	2.9	9	3.1	3.6
1999	91	3.5	3.8	20	6.9	7.2
2000	65	2.5	2.9	10	3.4	4.2
2001	81	3.1	3.5	15	5.2	5.3

2002	73	2.7	3.1	15	5.2	5.1
2003	69	2.6	2.7	10	3.4	3.3
2004	72	2.7	2.9	14	4.8	5.1
2005	80	3.0	3.2	18	6.2	6.1
2006	90	3.3	3.5	21	7.2	7.0
2007	93	3.4	3.6	14	4.8	4.4
2008	84	3.1	3.1	18	6.2	5.9
2009	97	3.5	3.6	14	4.8	4.7
2010	98	3.6	3.9	19	6.5	6.5
2011	107	3.9	3.9	24	8.2	7.3
2012	99	3.6	3.4	14	4.8	4.3
2013	93	3.3	3.3	18	6.2	5.2
2014	100	3.6	3.6	10	3.4	3.2
2015	112	3.9	3.8	23	7.8	6.6

Appendix B. STUDY II

Table 1a. Parishes in the Region of North Denmark with MM cases (all types of asbestos exposure) in 1974-2015 for women.

Parish	MM cases (N)	Female residents (N, median)	Cases per 100,000 residents (N)	RR for MM Parish/ Denmark	95% CI
1	11	3721	296	10.5	5.5, 19.4
2	7	6381	110	3.9	1.7, 8.4
3	5	4048	124	4.4	1.6, 10.9
4	5	3026	165	5.9	2.2, 14.5
5	4	3522	114	4.0	1.3, 11.1
6	4	4372	92	3.2	1.0, 8.9
7	4	6243	64	2.3	0.7, 6.3
8	3	2643	114	4.0	1.0, 12.8
9	2	6132	33	1.2	0.2, 3.7
10	2	3004	67	2.4	0.4, 9.5
11	2	4808	42	1.5	0.3, 5.9
12	2	1651	121	4.3	0.7, 17.3
13	2	1524	131	4.7	0.8, 18.7
14	2	3277	61	2.2	0.4, 8.7
15	2	4639	43	1.5	0.3, 6.2
16	2	2486	81	2.9	0.5, 11.5
17	1	4496	22	0.8	0.04, 5.1
18	1	1302	77	2.7	0.1, 17.7
19	1	483	207	7.4	0.4, 47.4
20	1	184	544	19.3	1.0, 122.6
21	1	574	174	6.2	0.3, 39.9
22	1	221	453	16.0	0.8, 102.4
23	1	2005	50	1.8	0.1, 11.5
24	1	163	614	21.8	1.1, 138.0
25	1	4122	24	0.9	0.05, 5.6
26	1	1746	57	2.0	0.1, 13.2
27	1	2981	34	1.2	0.1, 7.7
28	1	2088	48	1.7	0.1, 11.0
29	1	3405	29	1.0	0.1, 6.8
30	1	386	259	9.2	0.5, 59.1

31	1	413	242	8.6	0.5, 55.2
32	1	838	119	4.2	0.2, 27.4
33	1	3253	31	1.1	0.1, 7.1
34	1	5157	19	0.7	0.04, 4.5
35	1	91	1105	39.2	2.0, 243.8
36	1	1154	87	3.1	0.2, 20
37	1	1794	56	2	0.1, 12.8
38	1	257	390	13.8	0.7, 88.5
39	1	1573	64	2.3	0.1, 14.6
40	1	1862	54	1.9	0.1, 12.4
41	1	2335	43	1.5	0.1, 10
42	1	1654	61	2.1	0.1, 14
43	1	2506	40	1.4	0.1, 9.2
44	1	4007	25	0.9	0.04, 5.7

Table 1b. Parishes in the Region of North Denmark with MM cases (exclusively environmental asbestos exposure) in 1974-2015 for women.

Parish	MM cases (N)	Female residents (N, median)	Cases per 100,000 residents (N)	RR for MM Parish/ Denmark	95% CI
1	3	3721	80.6	2.9	0.7, 9.1
2	4	6381	62.7	2.2	0.7, 6.1
3	1	4048	24.7	0.9	0.04, 5.7
4	3	3026	99.2	3.5	0.9, 11.2
5	1	3522	28.4	1.0	0.1, 6.5
6	3	4372	68.6	2.4	0.6, 7.8
10	1	3004	33.3	1.2	0.1, 7.7
13	1	1524	65.6	2.3	0.1, 15.1
29	1	3405	29.4	1.0	0.1, 6.8
33	1	3253	30.7	1.1	0.1, 7.1
41	1	2335	42.8	1.5	0.1, 9.9

Table 2a. Parishes in the Region of North Denmark with MM cases (non-occupational asbestos exposure) in 1970-2015 for men.

Parish number	MM cases (N)	Male residents (N, median)	Cumulative incidence per 100,000 person-years	95% CI
2	7	6294	2.42	1.19, 5.86
3	5	3609	3.01	1.24, 8.37
5	3	3186	2.05	0.59, 7.31
6	3	4273	1.53	0.44, 5.45
301	3	2477	2.63	0.76, 9.40
1	2	3588	1.21	0.24, 5.48
10	2	2971	1.46	0.29, 6.61
250	2	272	15.98	3.11, 71.22
141	2	3436	1.27	0.25, 5.72
27	2	2844	1.53	0.30, 6.91
33	2	3000	1.45	0.28, 6.55
17	1	4399	0.49	0.03, 3.60
12	1	1709	1.27	0.08, 9.25
40	1	1738	1.25	0.07, 9.10
61	1	3666	0.59	0.03, 4.32
29	1	3278	0.66	0.04, 4.83
15	1	4486	0.48	0.03, 3.53
41	1	2315	0.94	0.06, 6.83
9	1	5644,5	0.39	0.02, 2.80
14	1	3132	0.69	0.04, 5.05
7	1	6319	0.34	0.02, 2.50
21	1	594,5	3.66	0.21, 26.47
154	1	626	3.47	0.20, 25.14
99	1	1225	1.77	0.10, 12.89
26	1	1675	1.30	0.08, 9.43
161	1	88	24.70	1.45, 172.06
172	1	337	6.45	0.38, 46.45
239	1	311	7.00	0.41, 50.36
266	1	5396	0.40	0.02, 2.93
316	1	1511	1.44	0.09, 10.46
332	1	454	4.79	0.28, 34.62
349	1	244	8.91	0.22, 63.86
44	1	3721	0.58	0.03, 4.25

Table 2b. Parishes in the Region of North Denmark with MM cases (occupational asbestos exposure) in 1970-2015 for men.

Parish number	MM cases (N)	Male residents (N, median)	Cumulative incidence per 100,000 person years	95% CI
2	22	6294	7.60	5.48, 13.13
3	20	3609	12.05	8.49, 21.25
130	16	4991	6.97	4.63, 12.99
17	12	4399	5.93	3.61, 11.97
1	11	3588	6.67	3.93, 13.81
5	10	3186	6.82	3.89, 14.56
10	8	2971	5.85	3.06, 13.48
6	8	4273	4.07	2.12, 9.38
12	8	1709	10.18	5.31, 23.41
40	8	1738	10.01	5.22, 23.01
61	7	3666	4.15	2.04, 10.05
29	6	3278	3.98	1.81, 10.23
15	6	4486	2.91	1.33, 7.48
41	6	2315	5.63	2.57, 14.48
4	6	2872	4.54	2.07, 11.68
119	5	862	12.62	5.22, 34.92
8	5	2525	4.30	1.78, 11.96
25	4	3949	2.20	0.79, 6.79
301	4	2477	3.51	1.26, 10.81
9	3	5645	1.16	0.33, 4.13
24	3	181	36.13	10.49,
14	3	3132	2.08	0.60, 7.44
250	3	272	23.98	6.96, 84.35
306	3	5428	1.20	0.355, 4.29
43	3	2438	2.68	0.78, 9.55
60	2	591	7.36	1.43, 33.08
75	2	669	6.50	1.26, 29.22
90	2	1956	2.22	0.43, 10.04
141	2	3436	1.27	0.25, 5.72
11	2	46376	0.94	0.18, 4.24
209	2	16345	2.66	0.52, 12.01
7	2	63195	0.69	0.13, 3.11
215	2	39865	1.09	0.21, 4.93
13	2	20295	2.14	0.42, 9.68

295	2	3210	1.35	0.26, 6.12
49	1	11085	1.96	0.11, 14.25
18	1	1211	1.80	0.10, 13.04
63	1	1813	1.20	0.07, 8.72
65	1	9385	2.32	0.14, 16.82
66	1	374	5.81	0.34, 41.90
21	1	5955	3.66	0.21, 26.47
73	1	15185	1.43	0.08, 10.41
79	1	2485	0.87	0.051, 6.37
103	1	602	3.61	0.21, 26.16
109	1	628	3.46	0.20, 25.06
123	1	389	5.59	0.33, 40.30
134	1	429	5.07	0.30, 36.62
154	1	626	3.47	0.20, 25.14
155	1	223	9.77	0.57, 69.92
156	1	1443	1.51	0.09, 10.95
27	1	2844	0.76	0.04, 5.56
167	1	3214	0.68	0.04, 4.92
192	1	560	3.89	0.23, 28.11
31	1	458	4.75	0.28,34.29
255	1	355	6.12	0.36, 44.12
33	1	3000	0.72	0.04, 5.27
284	1	928	2.34	0.14, 17
296	1	209	10.40	0.61, 74.35
36	1	1183	1.84	0.11, 13.35
299	1	2171	1.00	0.06, 7.28
37	1	1854	1.17	0.07, 8.53
321	1	290	7.50	0.44, 53.87
324	1	787	2.76	0.16, 20.03
325	1	780,5	2.79	0.16, 20.19
331	1	726	3.00	0.18, 21.71
31	1	458	4.75	0.28, 34.29
36	1	1183	1.84	0.11, 13.35

Appendix C. STUDY III

Table 1. Genes (N=147) screened in fresh frozen paraffin embedded tumors by The University of Chicago Medicine OncoPlus, a custom targeted genomic capture and next generation sequencing assay

ABL1	*ATM*	*ALK*	*APC*	*ARID1A*	*ARID2*	*ASXL1*	*ATM*	*ATR*	*ATRX*
AXL	*B2M*	*BAP1*	*BCOR*	*BCORL1*	*BIRC3*	*BLM*	*BRAF*	*BRCA1*	*BRCA2*
BTK	*CALR*	*CBL*	*CBLB*	*CCND1*	*CCND2*	*CCND*	*CDH1*	*CDKN2A*	*CEBPA*
CHEK1	*CHEK2*	*CSF1R*	*CSF3R*	*CTCF*	*CTNNA1*	*CTNNB1*	*CUX1*	*CXCR4*	*DAXX*
DDR2	*DDX3X*	*DDX41*	*DICER1*	*DNMT3A*	*EGFR*	*EP300*	*EPHA3*	*EPHA5*	*ERBB2*
ERBB3	*ERBB4*	*ERCC3*	*ESR1*	*ETV6*	*EZH2*	*FANCA*	*FAT3*	*FBXW7*	*FGFR1*
FGFR2	*FGFR3*	*FH*	*FLT3*	*FOXL2*	*GATA1*	*GATA2*	*GNA11*	*GNAQ*	*GNAS*
GRIN2A	*H3F3A*	*HIST1H3B*	*HIST1H3C*	*HNF1A*	*HRAS*	*IDH1*	*IDH2*	*IKZF1*	*ITPKB*
JAK2	*KDM6A*	*KDR*	*KIT*	*KMT2A*	*KRAS*	*MAP2K1*	*MAPK1*	*MET*	*MLH1*
MLH3	*MPL*	*MRE11A*	*MSH2*	*MSH6*	*MTOR*	*MYD88*	*NBN*	*NF1*	*NF2*
NFE2L2	*NOTCH1*	*NOTCH2*	*NPM1*	*NRAS*	*PALB2*	*PBRM1*	*PDGFRA*	*PDGFRB*	*PHF6*
PIK3CA	*PIK3CB*	*PIK3R1*	*PLCG2*	*POLE*	*POT1*	*PPP2R1A*	*PTCH*	*PTEN*	*PTPN11*
RAD21	*RAD51*	*RB1*	*RET*	*RUNX1*	*SDHB*	*SDHC*	*SDHD*	*SETBP1*	*SF3B1*
SMAD4	*SMARCB1*	*SMC1A*	*SMC3*	*SMO*	*SFSR2*	*STAG2*	*STK11*	*TERT (promot*	*TET2*
TP53	*TSC1*	*TSC2*	*U2AF1*	*VHL*	*WT1*	*ZRSR2*			

Table 2. Detailed clinical characteristics of patients with malignant mesothelioma and a germline cancer predisposition mutation.

Patient ID	Sex	Age at Diagnosis	Site of Origin	Histology	Second Cancer	Cancer in FDR or SDR	*Fulfilled criteria for genetic testing	Asbestos Exposure	Germline Gene Mutated	Acquired mutation(s)
Moderate to high penetrance risk alleles										
UC170	Female	57	Peritoneum	Epithelioid	Breast	Uterus, Lung, Colorectal, Prostate	Family history meets Lynch syndrome criteria	None	*ATM*	*BAP1* (p.Val247fs*2)
UC258	Male	59	Pleura	Epithelioid	None	Lung, Breast, Basal cell skin cancer	Personal + family history meet BAP1 criteria	Definite / Primary	*ATM*	NA
UC041	Male	37	Peritoneum	Epithelioid	None	Leukemia	No	Probable / Secondary	*BAP1*	*BAP1* (c.68-2A>C in trans) BAP1 loss by IHC
UC102	Male	65	Peritoneum	Epithelioid	None	Ovarian, Lung (3), Urinary tract	Family history meets HBOC criteria	Definite / Primary	*BAP1*	*BAP1* (c.437+2T>A) CSF1R (p.Leu

										756fs*23)
UC060	Male	61	Peritoneum	Epithelioid	None	Breast, Lymphoma, Lung, MM, Hepatic, Uterus	Personal + family history meet BAP1 criteria & family history meets HBOC criteria	Definite / Primary	*BAP1*	NA BAP1 loss by IHC
UC049	Male	61	Pleura and peritoneum	Unknown	Melanoma	MM(2), Renal(2), Basal cell skin cancer	Personal + family history meet BAP1 criteria	Possible / Secondary	*BAP1*	NA
UC221	Female	55	Peritoneum	Epithelioid	None	MM	Personal + family history meet BAP1 criteria	None	*BAP1*	NA
UC238	Female	74	Peritoneum	Epithelioid	Breast	Colorectal	Personal history meets HBOC criteria	None	*BAP1*	NA

UC264	Male	75	Pleura	Epithelioid	None	Lung, Liver	No	Probable / Primary	*BRCA1*	NA
UC169	Male	65	Pleura	Epithelioid	None	Pancreas, Breast, Melanoma	Personal + family history meet BAP1 criteria	Probable / Secondary	*BRCA2*	NA
UC191	Female	72	Pleura	Epithelioid	None	Breast	Family history meets HBOC criteria	None	*BRCA2*	NA
UC241	Female	56	Peritoneum	Epithelioid	None	Breast	No	None	*BRCA2*	NA
UC061	Female	32	Peritoneum	Epithelioid	Melanoma	Breast(2), Pancreas, Colorectal (3), Lung, Lymphoma	Family history meets HBOC criteria ** & Personal history meets BAP1 criteria	Possible / Primary & Secondary	*CDKN2A*	NA
UC2	Male	64	Pleura	Epithelioid	None	Head & Neck, Colorectal,	No	None	*CDKN2A*	NA

65						Esophageal				
UC0166	Male	61	Pleura	Epithelioid	None	Lymphoma, Colorectal	No	Probable / Primary & Secondary	*CHEK2*	None BAP1 loss by IHC
UC0644	Male	62	Pleura	Epithelioid	None	Lymphoma	No	Definite / Primary	*CHEK2*	NA
UC1299	Male	76	Peritoneum	Epithelioid	Colorectal, prostate	None	No	Definite / Primary	*CHEK2*	NA
UC2011	Male	66	Pleura	Biphasic	None	Unknown	No	Definite / Primary	*MRE11A*	NA
UC0811	Male	56	Pleura	Epithelioid	None	Melanoma (2), Lung, Colorectal	Personal + family history meet BAP1 criteria	Definite / Primary & Secondary	*MSH6*	MSI stable MMR proteins all intact by IHC
UC2422	Female	71	Peritoneum	Epithelioid	Breast, ovarian, GIST	Lung, Breast (2)	Personal history meets HBOC criteria ***	Possible / Secondary	*SDHA*	NA

UC049	Male	61	Pleura and peritoneum	Unknown	Melanoma	MM(2), Renal(2), Basal cell skin cancer	Personal + family history meet BAP1 criteria	Possible / Secondary	*TMEM127*	NA
UC124	Female	27	Pleura	Epithelioid	None	Prostate (4), Gastric	No^	None	*TP53*	NA
UC240	Female	78	Pleura	Epithelioid	None	Prostate, Hepatic	No	None	*VHL*	NA
UC059	Male	37	Peritoneum	Epithelioid	Wilm's tumor	Colorectal, Lung (2), Brain	No****	None	*WT1*	*BAP1* (p.Lys425fs*5) Complex karyotype on tumor cytogenetics
Low penetrance risk alleles										
UC139	Female	57	Pleura	Epithelioid	None	Lung, Breast(2)	No	None	*APC*	NA
UC2	Female	66	Pleura	Biphasic	None	Breast (2)	Family history meets	Definite /	*APC*	NA

30							HBOC criteria	Secondary		
UC102	Male	65	Peritoneum	Epithelioid	None	Ovarian, Lung (3), Urinary tract	Family history meets HBOC criteria	Definite / Primary	*MITF*	*CSF1R* (p.Leu756fs*23) *BAP1* (c.437+2T>A)
UC114	Female	60	Pleura	Epithelioid	None	Leukemia, Multiple myeloma	No	Definite / Primary & Secondary	*MITF*	NA
UC249	Male	77	Pleura	Epithelioid	Prostate	Lymphoma, Lung	No	Definite / Primary & Secondary	*MUTYH*	*BAP1* (p.Gln684*) *DDX3X* (p.Gln360*)

Abbreviations: FDR=first-degree relative; SDR=second-degree relative; NA=not available; HBOC= hereditary breast and ovarian cancer syndrome; MM=malignant mesothelioma; GIST=gastrointestinal stromal tumor; IHC=immunohistochemistry

**Family history is as obtained at the time of first patient interview. Clinical criteria for germline genetic testing include: National Comprehensive Cancer Network Genetic/Familial High-Risk Assessment: Breast and Ovarian and Colon Cancer guidelines (www.nccn.org) as well as BAP1 Tumor Prediposition Syndrome clinical testing recommendations (https://www.ncbi.nlm.nih.gov/books/NBK390611/)*

***This patient's family had a known BRCA2 mutation in the family, which UC061 did not carry, as well as the CDKN2A mutation identified here*

****This patient had clinical panel based testing for hereditary breast and ovarian cancer genes that was negative; this panel did not include SDHA.*

*****This patient had subtle hemihypertrophy and proteinuria*

^This patient had prior clinical genetic testing identifying the same TP53 mutation confirmed in our study

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-406-5

AALBORG UNIVERSITY PRESS